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(54) **CANCEROUS METASTASIS-ASSOCIATED GENE**

(57) A gene exhibiting stronger expression in mouse IMC-HM cells than in mouse IMC-LM cells and isolated by using the differential display method. When the relation between the expression of the isolated gene and cancerous metastasis is examined, it is found out that a transformant with the larger expression dose of the antisense mRNA to the gene shows the more lowered cancerous metastasis ability. By using the isolated protein, cancerous metastasis inhibitors can be screened.

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**Description**Technical Field

5 [0001] The present invention relates to a protein associated with cancer metastasis and a gene encoding the same.

Background Art

[0002] In spite of the remarkable development of methods of diagnosis and treatment, cancer is still one of the main  
 10 causes of death in most developed countries, including Japan. In malignant cancer, cancer cells not only randomly proliferate but also invade (infiltrate) surrounding organs and metastasize into distant organs. In fact, the majority of deaths due to cancer are caused by its recurrence by metastasis. Therefore, it seems possible to completely cure cancer by the surgical excision of cancerous foci if the metastasis or invasion of cancer cells is effectively suppressed. Now that a method for treating localized cancer cells is about to be established, the most preferred subject for improving the treatment of cancer is now likely to shift to how to treat metastasis. Hence, analyses of genes associated with the invasion  
 15 and metastasis of cancer cells and proteins as gene products thereof are extremely important for developing new cancer treatment methods.

[0003] Hitherto, a number of cancer metastasis-associated genes have been reported. For example, "nm23" was discovered by the differential hybridization method in mouse melanoma K-1735 subtypes exhibiting different colony forming abilities when they are transplanted through mouse tail vein to the lung. Its metastasis potency and expression in  
 20 Northern blot are inversely correlated. Transfer of cDNA of "nm23" into a highly metastatic cell clone has been reported to result in the reduction of experimental metastasis potency (cf. Steeg, P. S. et al., J. Natl. Cancer Inst., 80, 200-204 (1988) and Leone, A. et al., 25-35 (1991)). Also, a gene highly expressed in low metastatic cell lines was isolated by the differential hybridization method performed with low and high metastatic cells derived from rat prostate cancer R-3327 cell line; said gene was reported to encode fibronectin. In addition, a 4- to 8-fold reduction of the expression of mRNA of fibronectin was reported in many cell clones which became highly metastatic by allowing said clones to express the v-Ha-ras gene (cf. Schalken, J. A. et al., Cancer Res., 48, 2042-2046 (1988)). Furthermore, a number of genes including KAI1 (Dong, J. T. et al., Science, 268, 884-886 (1995)), stromelysin-3 (Basset, P. et al., Nature, 348, 699-704 (1990)), pGM21 (Phillips, S. M. et al., J. Natl. Cancer Inst., 82, 199-203 (1990)), etc. have been reported as cancer  
 25 metastasis-associated genes.

[0004] However, most of these reports were limited only to studies with the reduction of migration and invasion abilities of cancer cells, in which the expression of a target gene had been suppressed, as indicators. No report has appeared on the suppression effect actually demonstrated in the assay system using experimental animals.

35 Disclosure of the Invention

[0005] An objective of the present invention is to provide a novel protein associated with cancer metastasis and DNA encoding said protein. Another objective of this invention is to provide a vector carrying said DNA, a transformant harboring said vector, and a method for preparing a recombinant protein that comprises culturing said transformant. Still  
 40 another objective of this invention is to provide DNA used for detecting, isolating, and amplifying said gene, or for suppressing the expression thereof. A further objective of this invention is to provide a method of screening a cancer metastasis inhibitor using proteins and genes associated with cancer metastasis.

[0006] Mouse IMC-HM cells, mutant cells isolated from mouse IMC cell line by Arakawa et al., are highly metastatic to the liver. When IMC-HM cells are subcutaneously transplanted to the ventral of mice, first they spontaneously metastasize to the liver, then rapidly metastasize to the main organs of the whole body, leading to the death of the host in  
 45 about two weeks. In contrast, almost no metastasis potency is observed with IMC-LM cells, a subculture of the parental IMC cells. Mice transplanted with said cells survive for at least six weeks after the transplantation, and no metastatic foci are observed at all with the naked eye (cf. Arakawa, H. et al., Jap. J. Cancer Res., 87, 518-523 (1996)). Although it may be readily assumed that these two cell lines share a very close genetic background, they widely differ in properties such as the presence or absence of spontaneous metastasis potency to distant organs. In view of these facts, the present inventors suspected the presence of a key gene controlling cancer metastasis in mouse IMC-HM cells, and attempted to isolate a strongly expressed gene from mouse IMC-HM cells as compared with mouse IMC-LM cells using the differential display method. As a result, the present inventors succeeded in isolating two mouse cDNA fragments, which are candidates for the factor inducing cancer metastasis. Furthermore, they screened a cDNA library derived  
 50 from mouse IMC-HA1 Cells using these cDNA fragments as probes, and succeeded in isolating full-length cDNA thereof

[0007] The present inventors also investigated the relation between the expression of isolated mouse cDNAs and cancer metastasis. Specifically, they constructed a vector expressing antisense-mRNA against one of the isolated mouse

cDNAs (mouse CMAP), and expressed said vector in IMC-HA1 cells derived from IMC-HM cells to prepare many transformants which exhibited different expression levels of the antisense mRNA. The inventors then transplanted the transformants into mice to investigate their cancer-metastasis abilities. As a result, it was found that the higher the antisense MRNA expression level of the transformant is, the lower its cancer metastasis potency is. The inventors also succeeded in isolating human cDNA (human CMAP) that exhibited a high homology with mouse CMAP by the polymerase chain reaction using primers prepared based on the nucleotide sequence of isolated mouse CMAP.

[0008] Since a close relation was recognized between the expression of isolated genes and cancer metastasis, the present inventors found it was possible to screen cancer metastasis inhibitors using these proteins and genes.

[0009] The present invention relates to a novel protein and gene associated with cancer metastasis, and to a method of using them to screen cancer metastasis inhibitors. More specifically, the present invention relates to:

(1) a protein comprising any one of the amino acid sequences set forth in SEQ ID NOs: 4, 6, 9, or 38, or a protein comprising any one of said amino acid sequences having substitution, deletion, or addition of one or more amino acids and having cancer metastasis potency;

(2) a protein encoded by DNA which hybridizes with DNA comprising any one of the nucleotide sequences set forth in SEQ ID NOs: 3, 5, 7, 8, or 37, said protein having cancer metastasis potency;

(3) a DNA encoding the protein according to (1);

(4) the DNA according to (3) comprising any one of the nucleotide sequences set forth in SEQ ID NOs: 3, 5, 7, 8, or 37;

(5) a DNA hybridizing with a DNA comprising any one of the nucleotide sequences set forth in SEQ ID NOs: 3, 5, 7, 8, or 37, encoding a protein having cancer metastasis potency;

(6) a vector carrying the DNA according to any one of (3) to (5);

(7) a transformant harboring the vector according to (6);

(8) a method for preparing the protein according to (1) or (2), comprising culturing the transformant set forth in (7);

(9) a DNA specifically hybridizing with the DNA according to any one of (3) to (5), comprising at least 15 nucleotide residues;

(10) an antisense DNA against the DNA according to any one of (3) to (5) or a portion thereof;

(11) an antibody binding to the protein according to (1) or (2);

(12) a method for screening a compound having cancer metastasis inhibitory ability, comprising steps of:

(a) contacting a test sample with the protein according to (1) or (2), and

(b) selecting compounds having the activity to bind to the protein according to (1) or (2);

(13) a method for screening compounds having cancer metastasis inhibitory ability, comprising steps of:

(a) contacting test samples with cells expressing the protein according to (1) or (2),

(b) detecting the expression level of the protein according to (1) or (2) in cells contacted with test samples using the antibody set forth (11), and

(c) selecting a compound which reduces the expression level of the protein according to (1) or (2) as compared with that in cells not contacted with test samples.

[0010] The present invention relates to a novel protein associated with cancer metastasis. The nucleotide sequence of cDNA isolated and designated "CMAP" by the present inventors is shown in SEQ ID NO: 3, and the amino acid sequence encoded by said cDNA is shown in SEQ ID NO: 4. Mouse "CMAP" cDNA has been isolated by the differential display method as a gene which is highly expressed in a mouse cancer metastatic IMC-HM cell line derived from mouse IMC cells. Its expression was not detected in the IMC-LM cell line, which had been similarly derived from mouse IMC cell line but had almost no detectable cancer metastasis potency (Cf. Example 1). Studies on the expression of mouse CMAP in various cancer cells revealed that it was expressed in M-5076 cells, L5178Y cells, P388 cells, and L1210 cells, but not in IMC-HA1 cells, which formed metastasis foci mainly in the liver when transplanted through the tail vein of mice. In contrast, B-16-BL6 cells and Colon 26 cells, in which the expression of mouse CMAP was not detectable, strongly induced the experimental metastatic foci in the lung, but did not exhibit hepatic metastasis. All other cell lines in which mouse CMAP was expressed below the detection limit were almost completely rejected and regressed when they were transplanted to the tail vein (cf. Example 7). Many transformants that expressed antisense mRNA against a transcript of mouse CMAP cDNA in various levels were prepared and their cancer metastasis abilities *in vivo* were examined by transplanting them into mice. As a result, it was found out that higher expression levels of antisense mRNA of transformant were related with lower cancer metastasis potencies of said transformant (cf. Example 9). These facts indicate a close association of mouse CMAP expression with cancer metastasis.

[0011] In addition, the present inventors isolated cDNA (human CMAP cDNA) exhibiting a high homology with mouse

CMAF from human spleen by polymerase chain reaction using primers prepared based on the nucleotide sequence of mouse CMAF cDNA. This cDNA's nucleotide sequence is shown in SEQ ID NO: 37, and the amino acid sequence of the protein encoded by said cDNA is shown in SEQ ID NO: 38. Human CMAF had a homology as high as 71.6% with mouse CMAF in the amino acid sequence (Cf. Example 8). This fact strongly indicates that human CMAF functions similarly to mouse CMAF.

[0012] Furthermore, as in the case of mouse CMAF, the inventors isolated three cDNA clones (#7.16411, #8.323, and #11.24413) that were overexpressed in mouse IMC-HM cells, by the differential display method using mouse IMC-HM cells and IMC-LM cells. Nucleotide sequences of three cDNA clones are shown in SEQ ID NOs: 5, 7, and 8. Comparison of these nucleotide sequences strongly indicated that "#7.16411" and "#11.24413" are produced when the #8.323 is processed differently. Clones "#7.16411" and "#11.24413" have an identical nucleotide sequence except for about 100 bp at the 5'-end, and were presumed to encode proteins of 131 and 126 amino acids with 20 to 30 different amino acids of the N-terminus. Amino acid sequences of proteins encoded by "#7.16411" cDNA and "#11.24413" cDNA are shown in SEQ ID NOs: 6 and 9, respectively. When the expression patterns of "#7.16411" and "#11.24413" in various cancerous and normal cells were examined, "#7.16411" was detected in almost all cancerous cells and normal tissues in spite of the difference in their expression level. However, "#11.24413" was detected only in cancer cells such as IMC-HA1 cells derived from mouse IMC-HM cells, and P388 cells, and in normal tissues such as the thymus, lung, and spleen and slightly in the peripheral blood (cf. Example 6). These results indicate that #8.323 to "#7.16411" are processed in the majority of normal tissues, and "#11.24413" is assumed to be expressed in specific cells. "#11.24413" may be a factor in providing IMC-HA1 cells with properties such as a rapid invasion of the whole body. The above-described characteristics indicate the possibility of using these proteins for screening compounds having cancer metastasis inhibitory ability.

[0013] The protein of this invention may be prepared as a recombinant protein as well as a natural protein using the genetic recombination technique by methods known to those skilled in the art. The natural protein, for example, can be prepared by subjecting extracts of cells or tissues that are expected to express the protein of the present invention to affinity chromatography using the antibody of the present invention described below. The recombinant protein may be prepared by culturing cells transformed with the DNA encoding the protein of this invention as described below.

[0014] The present invention also relates to a protein functionally equivalent to the above-described protein (mouse CMAF, human CMAF, #7.16411, and #11.24413). For example, a functionally equivalent protein can be isolated by a method of inducing the amino acid mutation in a protein. Such a method is known to those skilled in the art. Skilled persons would be able to prepare a mutant protein functionally equivalent to the natural protein by appropriately substituting amino acid(s) in the above-described protein (comprising any one of amino acid sequences set forth in SEQ ID NOs: 4, 6, 9, and 38) using Kunkel's method (Kunkel, T. A. et al., *Methods Enzymol.* 154, 367-382 (1987)), the double primer method (Zoller, H. J. and Smith, H., *Methods Enzymol.* 154, 329-350 (1987)), the cassette mutation method (Wells, et al., *Gene* 34, 315-23 (1985)), the megaprimer method (Sarkar, G. and Sommer, S. S., *Biotechniques* 8, 404-407 (1990)), etc. Amino acid mutation of a protein may also occur spontaneously. Such a mutant protein derived from a natural protein by substituting, deleting, or adding amino acids in its amino acid sequence, and proteins functionally equivalent to the natural protein are also included in the protein of the present invention. Here, "functionally equivalent" means that the protein has cancer metastasis potency. Cancer metastasis potency of the protein can be detected by, for example, suppressing the expression or function of said protein in metastatic cancer cells (for example, using the antisense technique), transplanting said suppressed cells into an animal body, and examining its cancer metastasis potency (Cf. Example 9). The extent of amino acid mutation in the functionally equivalent protein compared with the natural type protein is usually 10% or less of the total amino acids, preferably 10 amino acids or less, more preferably 3 amino acids or less, still preferably 1 amino acid.

[0015] Another method known in the art for isolating the functionally equivalent protein is the hybridization technique (Hanahan, D. and Meselson, H., *Method. Enzymol.* 100, 333-342 (1983) and Benton, W. D. and Davis, R. W., *Science* 196, 180-182 (1977)). Those skilled in the art can easily obtain a protein functionally equivalent to the above-described protein from DNA that is isolated based on the DNA sequences (SEQ ID NOs: 3, 5, 7, 8, and 37) encoding the above-described protein or a portion thereof and highly homologous with said DNA sequences. Such a protein encoded by DNA hybridizing with the DNAs encoding the above-described proteins and functionally equivalent to said proteins is also included in the protein of the present invention. Here, "functionally equivalent" means that the protein has cancer metastasis potency similarly as described above. Hybridization for isolating functionally equivalent proteins is usually performed in 6 x SSC and 40% formamide at 25°C and subsequent washing with 1 x SSC at 55°C. Preferably, hybridization is performed in 6 x SSC and 40% formamide at 37°C and washing with 0.2 x SSC at 55°C. More preferably, hybridization is performed in 6 x SSC and 50% formamide at 37°C and washing with 0.1 x SSC at 62°C. Those skilled in the art can obviously realize such stringent hybridization conditions by appropriately selecting the dilution rate of SSC, concentration of formamide, temperature, etc. Animals from which functionally equivalent proteins are isolated include human, mouse, rat, rabbit, sheep, cow, dog, and pig, but are not limited thereto. A DNA encoding the protein thus isolated usually has high homology with a nucleotide sequence of cDNA encoding the above-described protein.

(nucleotide sequences set forth in SEQ ID NOs: 3, 5, 7, 8, and 37). High homology means a sequence identity of at least 70% or more, preferably 80% or more, and more preferably 95% or more, at the nucleotide level.

[0016] In addition, the present invention relates to DNA encoding the above-described protein of the present invention (mouse CMAP, human CMAP, #7.16411, #11.24413, and a protein functionally equivalent thereto). There are no limitations in the type of the DNA of this invention as long as it can encode the protein of the invention. The DNA of this invention thus includes synthetic DNA as well as cDNA and genomic DNA. The DNA of this invention can be isolated by methods known in the art. For example, cDNA can be screened by labeling cDNA encoding the protein of this invention (for example, cDNA comprising any one of nucleotide sequences set forth in SEQ ID NOs: 3, 5, 7, 8, and 37) or a fragment thereof, RNA complementary to said cDNA, or a synthetic oligonucleotide comprising a portion of the nucleotide sequence of said cDNA with  $^{32}\text{P}$ , etc., and then hybridizing the labeled product with a cDNA library derived from cells or tissues expressing the protein of this invention. Alternatively, said cDNA can be cloned by synthesizing oligonucleotides corresponding to the nucleotide sequence of said cDNA, and amplifying them using a polymerase chain reaction with cDNA derived from appropriate cells or tissues as a template. Genomic DNA can be screened by hybridizing a genomic DNA library with a probe such as cDNA encoding the protein of the present invention (for example, cDNA comprising the nucleotide sequence of SEQ ID NO: 3, 5, 7, 8, or 37), a portion thereof, complementary RNA, or a synthetic oligonucleotide containing a part of the sequence of said DNA, which are labeled with  $^{32}\text{P}$  and the like. Alternatively, genomic DNA can be cloned by amplification using polymerase chain reaction with synthetic oligonucleotide primers having a sequence corresponding to the nucleotide sequence of the cDNA. Synthetic DNA can be prepared by, for example, chemically synthesizing oligonucleotides comprising a partial sequence of cDNA encoding the protein of the present invention (for example, cDNA comprising the nucleotide sequence set forth in SEQ ID NOs: 3, 5, 7, 8, or 37), annealing them to form a double strand, and ligating them using a DNA ligase (Khorana, H. G. et al., J. Biol. Chem. 251, 565-570 (1976); and Goeddel, D. V. et al., Proc. Natl. Acad. Sci. USA 76, 106-10 (1979)).

[0017] The DNA thus prepared is useful for producing a recombinant protein. The protein of the present invention can be prepared as a recombinant protein by inserting DNA encoding the above-described protein of this invention (for example, cDNA comprising the nucleotide sequence set forth in SEQ ID NOs: 3, 5, 7, 8, or 37) into an appropriate expression vector, introducing said vector into appropriate cells, culturing transformants thus obtained, and purifying a protein thus expressed. Specifically, when *Escherichia coli* is a host, a plasmid vector such as pET-3 (Rosenberg, A. H. et al., Gene 56, 125-35 (1987)), pGEX-1 (Smith, D. B. and Johnson, K. S., Gene 67, 31-40 (1988)), etc. can be used. *Escherichia coli* can be transformed by Hanahan's method (Hanahan, D., J. Mol. Biol. 166, 557-580 (1983)), the electroporation method (Dower, W. J. et al., Nucl. Acids Res. 16, 6127-6145 (1988)), etc. A recombinant protein can be synthesized in the form of a fused protein in which it binds to the tag of histidine residues, glutathione S-transferase (GST), etc. at the N-terminus, etc., and can be purified by binding said fused protein to metal-chelate resin, GST-affinity resin (Smith, M. C. et al., J. Biol. Chem. 263, 7211-7215 (1988)), etc. The desired protein can be developed from the fused protein by cleaving the fused protein with thrombin blood coagulating factor Xa, etc. When a fission yeast, *Schizosaccharomyces pombe*, is a host, pESP-1 (Lu, Q. et al., Gene 200, 135-144 (1977)) or the like plasmid vector is used. For example, yeast is transformed by the spheroplast method (Beach, D. and Nurse, P., Nature 290, 140 (1981)), lithium acetate method (Okazaki, K. et al., Nucl. Acids Res. 18, 6485-6489 (1990)), etc. When pESP-1 is used, a recombinant protein is synthesized as a fusion protein with glutathione S-transferase (GST) and is purified by binding the fusion protein to GST-affinity resin. The protein of interest can be separated from the fused protein by cleaving it with thrombin, blood coagulating factor Xa, etc. When mammalian cells such as CHO cells derived from Chinese hamster ovary, human HeLa cells, etc. are the host, pMSG (CLONTECH) or the like vector is used. The recombinant DNA can be introduced into mammalian cells by the calcium phosphate method (Graham, F. L. and van der Eb, A. J., Virology 52, 456-467 (1973)), DEAE-dextran method (Sussman, D. J. and Milman, G., Mol. Cell. Biol. 4, 1641-1643 (1984)), lipofection method (Felgner, P. L. et al., Proc. Natl. Acad. Sci. USA 84, 7413-7417 (1987)), electroporation method (Neumann, E. et al., EMBO J. 1, 841-845 (1982)), etc. When insect cells are the host, the baculovirus vector pBacPAK8/9 (Clontech) or similar vectors are used. For example, insect cells can be transformed according to methods as described in Bio/Technology, 6, 47-55 (1980).

[0018] The present invention also relates to DNA specifically hybridizing with the above-described DNA of the present invention and having at least 15 nucleotide residues. Here, "specifically hybridize" means that DNA does not cross-hybridize with other DNAs encoding other proteins under stringent conditions. Such DNA can be utilized as a probe for detecting and isolating DNAs encoding the protein of this invention, and as a primer for amplifying said DNAs.

[0019] The present invention also relates to antisense DNA against the DNA encoding the protein of the present invention or a portion thereof. Such antisense DNA is utilized to suppress the expression of the protein of the present invention. For example, it is possible to suppress the cancer metastasis ability of said cells by expressing such antisense DNA in cells with cancer metastasis potency to suppress the expression of the protein of this invention. In order to display the antisense effect, the antisense DNA is at least 15 bp or more, preferably 100 bp or more, and usually shorter than 800 bp, preferably shorter than 600 bp.

[0020] Furthermore, this invention relates to an antibody binding to the protein of the present invention. Antibodies

binding to the protein of the present invention can be prepared by methods known in the art (cf. "Shin Seikagaku Jikkenkoza 1, Proteins I, 389-406, Tokyo Kagaku Dojin"). Polyclonal antibodies can be prepared as follows. A suitable amount of the above-described protein or peptide is administered to animals to be immunized such as rabbits, guinea pigs, mice, or chickens. The antigen may be administered together with an adjuvant (such as FIS and FCA) to stimulate the antibody production. Immunization is usually performed every several weeks. Repeated immunization allows elevating the antibody titer. After the final immunization, antiserum can be obtained by collecting the blood from immunized animals. Polyclonal antibodies can be prepared by fractionating the antiserum using the ammonium sulfate precipitation, anion exchange chromatography and affinity chromatography with protein A or the immobilized antigen. Monoclonal antibodies can be prepared as follows. The protein of this invention or a partial peptide thereof is administered to animals to be immunized as described above. After the final immunization, the spleen or lymph node is excised from immunized animals. Antibody-producing cells contained in the spleen or lymph node are fused with myeloma cells using polyethylene glycol to prepare hybridomas. The hybridomas of interest are then screened and cultured. Monoclonal antibodies can be prepared from the culture supernatant and purified by fractionation using the ammonium sulfate precipitation, anion exchange chromatography and affinity chromatography with protein A or the immobilized antigen. Antibodies thus prepared can be used not only for affinity purification of the protein of this invention but also for diagnosis and antibody treatment of diseases (e.g. cancer) caused by the abnormal expression of the protein of this invention, and detection of the expression level of the protein. For antibody treatment, the antibody to be used is preferably a humanized antibody or human antibody. Humanized antibody, for example, a mouse-human chimeric antibody, can be prepared by isolating the antibody gene from mouse cells producing the antibody against the protein of this invention, recombining the gene of the constant region of H chain with that of the constant region of human IgE H chain, and introducing the resulting recombinant gene into mouse myeloma J558L cells (Neuberger, N. S. et al., Nature **314**, 268-270 (1985)). Human antibodies can be prepared by immunizing mice, whose immune system has been replaced with that of humans, with the protein of this invention.

[0021] The present invention also relates to a method for screening a compound with cancer metastasis suppressing potency using the protein of this invention. One embodiment of the screening method according to this invention comprises steps of (a) contacting the protein of this invention with test samples, and (b) selecting a compound that binds to the protein of this invention. Test samples used for the screening include purified proteins, expression products of genes (including gene library), extracts of tissues or cells, supernatant of cell cultures, synthetic low molecular weight compounds, and metabolites of microorganisms, but are not limited to them. Test samples used for the screening may be appropriately labeled with a radioactive substance, a fluorescent substance, etc., but are not limited to them. Binding of test samples with the protein of this invention can be detected by the label on a compound bound to the protein of this invention (for example, detecting the binding by the radioactivity or fluorescence intensity). When the TWO-hybrid system is used (Zervos et al., Cell **72**, 223-232 (1994) and Fritz et al., Nature **376**, 530-533 (1995)), the binding can be detected by examining the activity of the reporter gene. Besides the above-described TWO-hybrid system, various methods known to those skilled in the art can be applied to screening compounds binding to the protein of this invention. The methods include the affinity purification method using a column to which the protein of this invention is immobilized and various peptide display methods such as the phage display method (F. Parmlly and G. P. Smith, Gene, **73**, 305 (1988)).

[0022] Another embodiment of the screening method of this invention comprises steps of (a) contacting test samples with cells expressing the protein of this invention, (b) detecting the expression level of the protein of this invention in the cells contacted with said test samples using an antibody binding to the protein of this invention, and (c) selecting a compound that reduces the expression level of the protein of this invention as compared with that in cells not contacted with said test samples. In this screening method, there are no limitations in the type of cells to be treated with test samples, as long as they express the protein of this invention. Cells to be treated may thus include IMC-HM cells or cells derived from them such as M-5076 cells and L5178Y cells. Test samples may include purified proteins, expression products of genes (including libraries), extracts of tissues or cells, supernatants of cell cultures, synthetic compounds of low molecular weights, and metabolites of microorganisms but are not limited to these. Many known methods, including the ELISA method, immune precipitation method, and Western blotting method, are available without limitation for detecting the expression level of the protein of this invention using an antibody. The antibodies used are labeled for detecting the protein of this invention if required. Examples of labels include enzymes, radioactive substances, and fluorescent substances, but are not so limited. It is also possible to detect the protein of this invention without labeling the antibody of the present invention but by labeling the molecule specifically binding to the antibody of this invention such as a secondary antibody or protein A. A desired compound can also be screened by detecting the expression of mRNA as well as that of the protein described above. The expression of mRNA can be detected by, for example, reverse transcription PCR (RT-PCR). G3PDH can be used as a control. Compounds isolated by the above screening methods may be candidates for suppressing the activity of the protein of this invention. These compounds can be utilized as cancer metastasis inhibitors.

[0023] Effects of the compounds isolated by the above-described screening methods are preferably confirmed *in vivo*

as occasion demands. One embodiment of the *in vivo* detection method comprises steps of (a) contacting a compound isolated by the above-described screening method with cells with cancer metastasis potency, (b) transplanting said cells to a nonhuman test mammal, and (c) detecting the cancer metastasis in said nonhuman test mammal to determine whether said compound suppresses cancer metastasis. When mice are used as the test animal, mouse IMC-HM cells or those derived from them are preferably used as the cells with cancer metastasis ability. In this case, cells can be transplanted to mice subcutaneously or through the tail vein. Cancer metastasis can be detected by examining the formation of metastasis foci, for example, in the liver.

[0024] Another embodiment of the *in vivo* detection of cancer metastasis suppressing ability of the compound is the method of directly administering the test compound to mice. This method comprises steps of (a) administering a compound isolated by the above-described screening method to nonhuman test mammals, (b) transplanting cells with cancer metastasis potency to said nonhuman test mammals, and (c) detecting cancer metastasis in said nonhuman test mammals to determine whether said compound suppresses cancer metastasis. The compound can be administered orally, intraperitoneally, through the tail vein, subcutaneously, intracutaneously, or intramuscularly. As in the above-described method, when mice are used as the test animal, cells with cancer metastasis ability are preferably mouse IMC-HM cells or those derived therefrom. These cells can be transplanted to mice subcutaneously or through the tail vein, etc. Cancer metastasis can be detected by examining the formation of metastasis foci, for example, in the liver.

#### Brief Description of the Drawings

[0025]

Figure 1 is a photograph showing electrophoretic patterns of CMAP expressed in various cancer cells detected by Northern blotting.

Figure 2 is a photograph showing electrophoretic patterns of CMAP expressed in normal tissues detected by Northern blotting.

Figure 3 compares amino acid sequences between CMAP and human family-2 cystatin.

#### Best Mode for Implementing the Invention

[0026] The present invention will be described in more detail below with reference to examples, but is not to be construed to be limited to these examples.

#### Example 1 Cloning of cancer metastasis-associated gene

##### (1) Extraction of the total RNAs from IMC-HM and IMC-LM cells

[0027] The total RNAs were extracted from IMC-HM and IMC-LM cells using ISOGEN (Nippon Gene). First, cultured cells in the logarithmic growth phase ( $1 \times 10^7$  cells) were collected and sedimented by centrifugation. After the culture supernatant was removed by aspiration, ISOGEN (1 ml) was added to the cells, and the mixture was allowed to stand at room temperature for 5 min to lyse the cells. Chloroform (0.2 ml) was then added to the mixture, and the resulting mixture was vigorously vortexed for 15 sec, allowed to stand at room temperature for several minutes, and centrifuged ( $4^\circ\text{C}$ ) at  $12,000 \times g$  for 15 min. The aqueous layer containing RNA was collected, and an equal volume of isopropanol was added thereto. The mixture was stirred, allowed to stand at room temperature for 10 min, and centrifuged again under cooling at  $12,000 \times g$  for 15 min. RNA thus sedimented was collected, rinsed with ethanol, and dissolved in sterilized pure water (50  $\mu\text{l}$ ), which had been treated with diethyl pyrocarbonate (DEPC) to obtain a total RNA solution. When cultured cells ( $5 \times 10^4$  cells) from which the total RNA was isolated were transplanted subcutaneously into the ventral of a five-week old female CDF1 mouse, observation with the naked eye revealed that IMC-HM cells metastasized to the liver of the host mouse to kill it in two weeks, while IMC-LM cells did not metastasize to the liver, allowing it to live for six weeks.

##### (2) Preparation of single-stranded cDNA using reverse transcriptase

[0028] A complementary single-stranded DNA was prepared with the total RNA obtained above as a template using reverse transcriptase Superscript II (GIBCO BRL) derived from MMLV and an mRNA Fingerprinting Kit (Clontech). A cDNA synthesis primer (oligo dT primer) (1  $\mu\text{l}$ ) was added to the total RNA (2  $\mu\text{g}$ ), and the total mixture volume was made 5  $\mu\text{l}$  by adding DEPC-treated, sterilized pure water. The mixture was kept at  $70^\circ\text{C}$  for 3 min then quickly cooled. A mixed solution (total volume 5  $\mu\text{l}$ ) of the reaction buffer (5  $\times$  first strand buffer) (2  $\mu\text{l}$ ), dNTP mix (each 5 mM) (2  $\mu\text{l}$ ), and Superscript II (200 unit/ $\mu\text{l}$ ) (1  $\mu\text{l}$ ) was added to this mixture, and the resulting mixture was allowed to stand at  $42^\circ\text{C}$

for 1 hour in an air incubator. After the reaction was completed, the mixture was treated at 72°C for 10 min, diluted 160-fold with the sterilized pure water, and stored at -20 °C. The RNA fingerprinting was then performed based on polymerase chain reaction (PCR) using this single-stranded cDNA solution.

### 5 (3) Amplification of single-stranded cDNA by PCR

[0029] Equal amounts of LA Tag DNA polymerase (Takara Shuzo) (5 U/  $\mu$ l) and undiluted solution of TaqStart Antibody (registered trade mark, Clontech) were mixed, and the mixture was allowed to stand at room temperature for a few minutes to make a 50 x polymerase mix. This mix (0.4  $\mu$ l) was combined with 10 x LA PCR buffer (Takara Shuzo) (2  $\mu$ l),  
 10 sterilized pure water (13.9  $\mu$ l), dNTP mix (Clontech) (5 mM each) (0.2  $\mu$ l), and [<sup>35</sup>S]dATP $\alpha$ S (Amersham) (0.5  $\mu$ l), and the resulting mixture was used as a PCR master mix. To the stock solution of the single-stranded cDNA (1  $\mu$ l) were added 20  $\mu$ M of P primer (P1-10/SEQ ID NOs: 10 to 19), 20  $\mu$ M of T primer (T1-9/SEQ ID NOs: 20 to 28) (Clontech) (1  $\mu$ l each), and the above-described PCR master mix (17  $\mu$ l). The resulting mixture was overlaid with mineral oil (Sigma), and PCR was performed in a DNA thermal cycler PJ2000 (PERKIN ELMER CETUS) to amplify several genes at once.  
 15 The PCR comprises one cycle of 94°C for 5 min, 40°C for 5 min, and 68°C for 5 min; two cycles of 94°C for 2 min, 40°C for 5 min, and 68°C for 5 min; 25 cycles of 94°C for 1 min, 60°C for 1 min, and 68°C for 2 min; and finally 68°C for 7 min.

### (4) Separation of PCR products by electrophoresis

20 [0030] A plate for the sequence gel (20 x 40 cm) was assembled using a spacer according to the standard method. A 6% glycerol-resistant gel and glycerol-resistant buffer (United States Biochemical) were used. PCR products (3.5  $\mu$ l each) amplified with P primer and T primer were mixed with a reaction termination solution (2  $\mu$ l each), heated at 94°C for 2 to 3 min, and then cooled. The resulting mixture (3  $\mu$ l each) was loaded onto the glycerol-resistant gel and electrophoresed at 35 W for about 3 hours. After electrophoresis, the gel was transferred onto a filter paper, which was dried  
 25 at 80°C for 2 hours in a gel dryer. Radioactivity in the gel was copied on an imaging plate for BAS2000 (Fuji Film) by leaving it on said plate overnight, and imaged with BAS2000.

[0031] This imaging analysis was used to search for genes whose expression level significantly differed between IMC-HM cells and IMC-LM cells. If the expression level of one gene is predominantly high in IMC-HM cells, said gene can be a candidate of a gene that induces cancer metastasis. Furthermore, if the expression level of one gene is predominantly high in IMC-LM cells, said gene would be a candidate of a gene that suppresses cancer metastasis. Two  
 30 bands of possible candidates for the factor inducing metastasis were found from IMC-HM cells. One band was detected in PCR products obtained from a primer set of P2 and T3, and the other from a primer set of P2 and T8. The former band was designated "23-1," and the latter band, "28-1."

### 35 (5) Cloning of IMC-HM cell-specific cDNA bands

[0032] IMC-HM cell-specific cDNA bands 23-1 and 28-1 were excised directly from the dried gel, and each gel segment was boiled in sterilized pure water (40  $\mu$ l) for 10 min to elute the desired cDNA fragments. This minute quantity of cDNA fragment was re-amplified as follows.

40 [0033] 10 x EX Tag buffer (Takara Shuzo) (5  $\mu$ l), dNTP mix (each 2.5 mM) (1  $\mu$ l), EX Taq DNA polymerase (Takara Shuzo) (5 U/ $\mu$ l) (0.5  $\mu$ l), and sterilized pure water (31.5  $\mu$ l) were mixed to make a PCR master mix. The eluted cDNA fragment (7  $\mu$ l) and the P primer and T primer (2.5  $\mu$ l each) which had been used for detecting said cDNA band were added to this mix. The mixture was overlaid with mineral oil and subjected to PCR comprising 20 cycles of 94°C for 1 min, 60°C for 1 min, and 68°C for 2 min in a DNA thermal cycler PJ2000 (PERKIN ELMER CETUS) to specifically  
 45 amplify the desired cDNA fragment. PCR products were electrophoresed on 1% agarose gel using a Mupid (COSMO-BIO) at 50 V for 1 hour and identified by staining with ethidium bromide.

[0034] In general, PCR products of cDNA have additional A at their 3' -terminus, and may be directly cloned by a TA type vector (having T at its terminus). Therefore, cDNA fragments thus re-amplified were cloned using a TA cloning kit (Invitrogen) according to the following method. A 10 x ligation buffer (Invitrogen) (1  $\mu$ l), pCR II vector (registered trade  
 50 mark, Invitrogen) (25 ng/ml) (2  $\mu$ l), sterilized pure water (9  $\mu$ l), and T4 DNA ligase (Invitrogen) (1  $\mu$ l) were added to re-amplified PCR fragments (5  $\mu$ l), and the mixture was allowed to stand at 14°C overnight to insert the cDNA. A tube of One Shot INV $\alpha$ F<sup>+</sup> competent cells (registered trade mark, Invitrogen) was mixed with 0.5 M  $\beta$ -mercaptoethanol (50  $\mu$ l). The above-described vector previously ligated with cDNA (2  $\mu$ l) was added to this mixture, and the resulting mixture was incubated at 42°C for 30 sec. After standing on ice for 2 min, an SOC medium (450  $\mu$ l) was added, and the mixture  
 55 was shake-cultured at 37°C for 1 hour. The solution obtained after the above transformation procedure (100  $\mu$ l and 200  $\mu$ l) was spread onto a previously prepared LB agar plate containing 50  $\mu$ g/ml kanamycin and X-Gal and allowed to stand at 37°C overnight. The vector without the insertion reacts with X-Gal to turn blue while the one with cDNA incorporated turns white. According to this principle, 10 white colonies were picked up and incubated again to be used for



sequencing; at the same time, replicas were prepared. Plasmids were prepared from each transformant cell strain using a QIAprep Spin Plasmid Kit (QIAGEN) by the following procedure.

[0035] Each transformant cell strain was incubated in an LB medium (3 ml) containing 50 µg/ml kanamycin at 37°C for about 8 hours and centrifuged to collect the cells. These cells were dissolved in buffer P1 (QIAGEN) (250 µl), mixed with buffer P2 (QIAGEN) (250 µl), further mixed with buffer P3 (QIAGEN) (350 µl), and then centrifuged at 10,000 x g for 10 min. The supernatant was transferred onto a QIAprep Spin column (QIAGEN) and centrifuged at 10,000 x g for 1 min. The column was washed with buffer PB (QIAGEN) to remove the nuclease activity and further washed with the buffer PE (QIAGEN) (0.75 ml). The plasmid retained in the column was then eluted with the TE buffer and collected. The concentration of the plasmid was calculated from the A260 value determined with a Hitachi spectrophotometer (U-3300).

#### (5) Sequencing of the cloned IMC-HM cell-specific cDNA fragment

[0036] A nucleotide sequence of cDNA was determined by the dye terminator method (equipment used, ABI Model 377). Although performance conditions may vary depending on the purification grade and concentration of plasmid serving as a template, in general the sequence consisting of 500 to 600 nucleotides may be determined at the same time using the dye terminator method. pCR II vector has the forward and reverse primer sites of M13 near the insertion site. Using each primer previously fluorescence-labeled, both strands were compared to determine the respective sequences. AmpliTaq (25 µl), 5 x buffer (25 µl), and purified water (125 µl) were mixed to dilute the enzyme, and premix was prepared for each of A, C, G, and T. To prepare premix of A or C, equal volumes of d/ddNTP, dye-primer, 5 x buffer, and the diluted enzyme (25 µl each) were mixed; the premix of G or T was prepared by mixing 50 µl each of these ingredients. The template DNA (100 to 200 ng/µl) (1 µl) and the premixes of A and C (4 µl each), and said template DNA (2 µl) and the premixes of G and T (8 µl each) were separately mixed and subjected to cycle sequencing reaction with 25 cycles of 96°C for 2 min, 96°C for 10 sec, 50°C for 5 sec, and 60°C for 4 min using a PERKIN ELMER 9600. Four clones randomly selected from 10 each cloned plasmids were sequenced, and all of them were found to have identical nucleotide sequences. One of the plasmid clones obtained from 23-1 was then designated 23-1#2, and that obtained from 28-1 was designated 28-1#3.

[0037] Next, sequences determined for 23-1#2 (SEQ ID NO: 1) and 28-1#3 (SEQ ID NO: 2) were analyzed with a computer using software from Genetics Computer Group, Wisconsin Package version 8.1. GenBank and EMBL database searches for the sequence homology with a FastA revealed that these sequences were entirely novel and had never been reported.

#### Example 2 Establishing clonal lines reflecting properties of IMC-HM cells and IMC-LM cells

[0038] Clonal lines of IMC-HM cells and IMC-LM cells were established by the limiting dilution culture method. Cells of each strain were distributed on a 96-well plate to 4 cells/10 wells, and 10 clones that had each proliferated from a single cell were randomly selected.

[0039] In order to detect the expression of 23-1#2 and 28-1#3 in each of these clones, RT-PCR was performed as follows. The total RNA and the reverse transcriptase were prepared similarly to the above-described methods ((1) and (2) in Example 1). In order to amplify the specific cDNA by PCR, single-stranded oligo DNA primers, "23-1#2F1" (CAGAATCTGCTCATGCAGTC (SEQ ID NO: 29)), "23-1#2R1" (CACTCCTTACTTTCCACCCC (SEQ ID NO: 30)), "28-1#3F1" (AACAGCATTTCCCTAAAGCTCGG (SEQ ID NO: 31)), and "28-1#3R1" (TGGAAACTACTTCCCTGCTCCCA (SEQ ID NO: 32)) were designed.

[0040] Portions of the sequences of 23-1#2 and 28-1#3 can be specifically amplified using a set of 23-1#2F1 and 23-1#2R1 and another set of 28-1#3F1 and 28-1#3R1. Thirty cycles of PCR were performed using single-stranded cDNAs prepared from cells derived from IMC-HM and IMC-LM cells as a template, and EX Tag DNA polymerase under conditions similar to those in Example 1 (5). As a result, it was observed that both 23-1#2 and 28-1#3 were amplified as cDNAs of a similar appropriate size in the IMC-HM-derived clonal cells, while no signals were detected in the IMC-LM-derived clonal cells.

[0041] Next, in order to confirm the metastasis potency of each of the clonal cells *in vivo*, 5 x 10<sup>4</sup> cells were subcutaneously transplanted to CDF1 mice. All clonal cells derived from IMC-HM killed the host two to three weeks after the transplantation, and their remarkable metastasis was observed with the naked eye in the liver and spleen while no metastasis foci were observed at all with the cells derived from IMC-LM. In addition, no distinct difference was detected between the cells derived from IMC-LM and those derived from IMC-HM, even by the observation of morphology, proliferation ability *in vitro*, etc. One clonal cell strain each was selected from the IMC-HM- and IMC-LM-derived clonal cells and designated "IMC-HA1" and "IMC-LE5," respectively. The IMC-HA1 cell was deposited with the following depository institution:

(a) Name of the depositary institution and its address:

Name: National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology  
Address: 1-3, Higashi 1-chome Tsukuba-shi Ibaraki-ken 305-0046 Japan

(b) Date of deposition (Date of original deposition): April 1, 1997

(c) Accession Number: FERM BP-6242

**Example 3** Analysis of clones 23-1#2 and 28-1#3 in IMC-HA1 and IMC-LE5 cells by Northern blot

[0042] In order to examine whether the expression of clones 23-1#2 and 28-1#3 differs at the poly(A) RNA level, Northern blot analysis was performed. Poly(A) RNA was prepared from IMC-HA1 and IMC-LE5 cells using a Messenger RNA Isolation Kit (STRATAGENE) as follows.

[0043] About  $10^8$  cells were recovered from a culture suspension (100 ml) and dissolved in a denaturing solution (STRATAGENE) (5 ml) containing  $\beta$ -mercaptoethanol (50  $\mu$ l). An elution buffer (STRATAGENE) (10 ml) was added to this mixture, and the resulting mixture was centrifuged at 12,000 x g for 10 min. The supernatant was mixed with oligo-dT cellulose (0.04 g/ml) (5 ml) and gently stirred at room temperature for 15 min. After the mixture was centrifuged at 700 x g for 3 min, the supernatant was removed, and the cellulose was resuspended in a high-salt buffer (STRATAGENE) (5 ml). This washing process was repeated twice, and then the cellulose residue was suspended in a low-salt buffer (STRATAGENE) (5 ml). The cellulose suspension was then packed into a 2.5-ml push column (STRATAGENE), and the remaining poly(A) RNA was eluted with an elution buffer (400  $\mu$ l) at 68°C. 3 M sodium acetate (40  $\mu$ l) and 100% ethanol (1.1 ml) were added to the eluate, and the mixture was centrifuged at 15,000 x g. Precipitates thus obtained were rinsed with 80% ethanol and dissolved in a TE buffer containing no RNase (40  $\mu$ l) to make a poly(A) RNA solution. Concentration was determined by measuring the absorbance (A260).

[0044] Next, for Northern blot analysis, 23-1#2 and 28-1#3 were directly radio-labelled with [ $\alpha$ - $^{32}$ P]dCTP using a Megaprime DNA labelling system (Amersham) according to the standard method. The primer (5  $\mu$ l) was added to cDNA (100 ng) to make the total volume 33  $\mu$ l, and the mixture was heated at 95°C for 5 min and allowed to stand at room temperature. A labelling buffer (Amersham) (10  $\mu$ l), [ $\alpha$ - $^{32}$ P]dCTP (5  $\mu$ l), and Klenow DNA polymerase I (1 unit/ $\mu$ l) (2  $\mu$ l) were added to this mixture, and the mixture was incubated at 37 °C for 10 min. To terminate the reaction, 0.5 M EDTA (3.5  $\mu$ l) was added to this mixture, and the labelled probe and unreacted [ $\alpha$ - $^{32}$ P]dCTP were separated using a Sephadex G-50 spin column. Eluates from the column were diluted with purified water, and radioactivity of the probe was counted with a liquid scintillation counter.

[0045] Northern blot analysis was then performed. Poly(A) RNA (2  $\mu$ g) was loaded on a 1% agarose gel prepared in 1 x MOPS buffer and electrophoresed at 50 V for 1.5 hour. After the electrophoresis, the gel was left standing overnight to transfer the nucleic acid from the gel onto a Hybond N+ membrane (Amersham) via 10 x SSC buffer. After the transfer, the membrane was air-dried, and the nucleic acid on the membrane was fixed by UV with a STRATALINKER. After the UV treatment, hybridization was carried out as follows.

[0046] The membrane was soaked in a prehybridization solution (containing 5 x Denhardt's solution, 50% formamide, 2% SDS, and denatured salmon sperm DNA (100  $\mu$ g/ml)), and prehybridization was performed at 42°C for 1 hour. [ $\alpha$ - $^{32}$ P]dCTP-labelled 23-1#2 and 28-1#3 were then added to the above-described prehybridization solution as the probe, and said membrane was allowed to stand at 42°C overnight. After the hybridization, the membrane was washed once with 2 x SSC in 0.1% SDS solution at 50°C for 30 min and twice with 0.5 x SSC in 0.1% SDS solution at 50°C for 30 min. The radioactivity was imaged according to the above-described method. 23-1#2 and 28-1#3 exhibited bands of about 1.1 kb and 0.7 kb, respectively, which hybridized only with the bands from IMC-HA1 Cells.

**Example 4** Analysis of clones 23-1#2 and 28-1#3 in IMC-HA1 and IMC-LE5 cells by Southern blot

[0047] Genomic DNA was prepared from IMC-HA1 and IMC-LE5 cells using a DNA Extraction Kit (STRATAGENE) and digested with EcoRI. The digested products were loaded (10  $\mu$ g each) onto a 1% agarose gel prepared in a TAE buffer, separated by electrophoresis at 50 V, transferred to Hybond N+, and subjected to Southern blot analysis. Both cDNA probes exhibited identical band patterns. Therefore, it was indicated that both 23-1#2 and 28-1#3 were present in the genome of both IMC-HA1 and IMC-LE5 cells but expressed more predominantly in IMC-HA1 cells.

**Example 5** Cloning of full-length cDNAs of clones 23-1#2 and 28-1#3

(1) Preparation of cDNA library derived from IMC-HA1 cells

[0048] A cDNA library derived from IMC-HA1 cells was prepared using a ZAP Express cDNA Synthesis Kit (STRAT-

AGENE) by the following method.

(a) Preparation of the total RNA and poly(A) RNA

- 5 [0049] The total RNA was extracted from IMC-HA1 cells by the method described in Example 1 (1). In this case, extraction with ISOGEN was repeated twice to reduce the contamination of proteins, lipids, DNA fragments, etc. From this total RNA, poly(A) RNA was extracted as follows. A spin column was prepared by placing a Whatmann 3M filter paper on the bottom of a 2.5-ml syringe and filling it with 5 ml of oligo(dT)-cellulose (0.04 g/ml) (STRATAGENE). The column was loaded with a sample, allowed to stand at room temperature for 30 min, then washed three times with a high-salt buffer and a low-salt buffer (Clontech). After elution with an elution buffer (Clontech), a 1/5 volume of a sample buffer was added to the eluate, the resulting mixture was loaded onto a new column, and the same procedure was repeated. After precipitation with ethanol and rinse, the product was dissolved in a TE buffer to make a poly(A) RNA solution.

15 (b) Synthesis of the first strand cDNA

- [0050] Poly(A) RNA (5 µg) and XhoI linker primer (170 pmol) were mixed, treated at 70°C for 5 min, then cooled in ice. 5 x first strand buffer (10 µl), 0.1 M dithiothreitol (GIBCO BRL) (5 µl), dNTP mix (10 mM each) (3 µl), and Rnasein (40 U/µl) (STRATAGENE) (1 µl) were added to this mixture, and reverse transcriptase Superscript II (GIBCO BRL) (12,000 U) was added to make a final volume of 50 µl. The resulting mixture was incubated at 44°C for 1 hour.

(c) Synthesis of the second strand cDNA

- 25 [0051] A 10 x second strand buffer (20 µl), second strand dNTP mix (STRATAGENE) (6 µl), sterilized pure water (113.9 µl), and [ $\alpha$ -<sup>32</sup>P]dATP (Amersham) (2 µl) were added to the first strand cDNA synthesized above (45 µl), and *E. coli* RNase H (3 U) and *E. coli* DNA polymerase (STRATAGENE) (100 U) were added. The mixture was incubated at 16°C for 2.5 hours to synthesize the second strand.

(d) Insertion of cDNA into the ZAP Express Vector

- 30 [0052] The free terminus of the XhoI linker primer was blunted with Pfu DNA polymerase (STRATAGENE) (5 U), ligated to the EcoRI adaptor with T4 DNA ligase (4 U) by allowing the mixture to stand at 8°C overnight, and further phosphorylated at the EcoRI terminus with T4 polynucleotide kinase (10 U). The XhoI linker terminus was cleaved by XhoI (120 U). cDNA was inserted into the ZAP Express Vector between the EcoRI and XhoI sites by reacting with the T4 DNA ligase at 12°C overnight.

(e) *In vitro* packaging and titer assay

- 40 [0053] cDNA was packaged into phage using a Gigapack Gold II (STRATAGENE) exactly according to the process recommended by the supplier's protocol. The titer of the phage solution after packaging was assayed as follows. The original phage solution and its 10-fold dilution with an SM buffer were prepared separately, and 1 µl each of both solutions was incubated with 200 µl of XL1-Blue MRF' (OD<sub>600</sub> = 0.5) at 37°C for 15 min to infect the host bacteria. Incubated mixtures were overlaid on a 100 mm NZY agar (GIBCO) plate together with 3 ml of an LB top agar, 0.5 M IPTG, and 12.5 mg of X-Gal, and allowed to stand overnight. Ninety-five percent or more of plaques were found to retain the insert, and the volume of phage solution required for obtaining 50,000 plaques was determined from the number of plaques formed.

(f) Amplification of cDNA library

- 50 [0054] Appropriate amounts of phage-infected host bacteria were overlaid on 20 previously prepared 150 mm NZY agar plates together with the top agar so that 50,000 plaques/plate could be obtained. These plates were then incubated at 37°C overnight to form plaques. After 8 ml of SM buffer was added, the plates were allowed to stand at 4°C overnight. The SM buffer in which phage was sufficiently released was recovered, and the plates were further rinsed with 2 ml of the same buffer to recover the phage. This was distributed in aliquots as the cDNA library stock solution and stored frozen at -70°C. This stock solution was used for screening the cDNA library.

## (2) Screening of cDNA library derived from IMC-HA1 cells

## (a) Primary screening

5 [0055] Twenty NZY agar plates were prepared in sterilized square #2 petri dishes. XL1-Blue MRF<sup>+</sup> adjusted to OD<sub>600</sub> = 0.5 was infected with the cDNA library phage so as to obtain 50,000 plaques/plate, overlaid on the above NZY agar plates together with an LB top agar, and allowed to stand at 37°C overnight. The next day, after suitable plaque formation was confirmed, the plates were cooled at 4°C for 2 hours, and DNA in plaques was transferred to a Hybond N+ membrane. The membrane was denatured, dried, UV cross-linked, then subjected to hybridization by the following procedure.

10 [0056] The membrane was soaked in a solution containing a 2 x PIPES buffer, 50% formamide, 0.5% SDS, and denatured salmon sperm DNA (100 µg/ml) and prehybridized at 42 °C for 1 hour. [α - <sup>32</sup>P]dCTP-labeled 23-1#2 and 28-1#3 were prepared according to the above-described method and added as probes to the prehybridization solution. The mixture was allowed to stand at 42°C overnight. After hybridization, the membrane was washed three to four times with a solution containing 0.1 x SSC and 0.1% SDS at 65°C for 20 min. The radioactivity was imaged according to the above-described method. Images were printed out in the actual size of the plate using a BAS 2000. Points corresponding to positive plaques on the plate were punched out with a sterilized and blunted tip of a P1000, and the phage was eluted into an SM buffer.

## 20 (b) Secondary screening

[0057] Phage solutions that had been judged positive in the primary screening were further screened by a procedure similar to that described above. In this case, a serial 10-fold dilution of the phage solution was prepared and overlaid on 100 mm plastic dishes in place of sterilized square #2 petri dishes. The phages localized at the point judged positive in the secondary screening were eluted into the SM buffer, and the DNA insert was amplified using a set of T7 and T3 primers. At this stage, when many inserts were detected, a similar screening process was continued.

[0058] This screening of the cDNA library yielded a phage clone #9.3133 that specifically binds to 23-1#2, and clones #7.1641, #8.32, and #11.2441 that specifically bind to 28-1#3 were obtained.

## 30 (c) Cloning into pBK-CMV phagemid vector

[0059] It is possible to directly excise out the ZAP Express vector *in vivo* as the pBK-CMV phagemid vector containing inserts in the presence of a helper phage (ExAssist helper phage). The pBK-CMV phagemid vector was excised out from the above-described phage clones, introduced into XL0LR cells, and allowed to form colonies on kanamycin-containing LB plates for cloning. Phagemid vector clones #9.31334, #7.16411, #8.323, and #11.24413 were thus obtained.

## (d) Sequencing of clones #9.31334, #7.16411, #8.323, and #11.24413

40 [0060] Partial sequencing of the clones was performed by the dye primer method using the pBK-CMV phagemid vector containing these cDNAs. Specifically; sequencing was performed similarly as described in Example 1 (6), except that the cycle sequencing reaction was carried out using T3 primer (AATTAACCCTCACTAAAGGG/SEQ ID NO: 33) or T7 primer (GTAATACGACTCACTATAGGGC/SEQ ID NO: 34). Furthermore, a primer to be used in RT-PCR and an appropriate primer were designed based on the sequence of the insert in the pBK-CMV phagemid vector whose cDNA sequence was partially determined by the dye primer method. The undetermined region was then sequenced by the dye terminator method using a PRISM Ready Reaction Terminator Cycle Sequencing Kit. Double-stranded DNA (250 to 500 ng), primer (3.2 pmol), and the above-described premix (8 µl) were mixed in a final volume of 20 µl, and the cycle sequencing was performed by the reaction of 96°C for 2 min, and 25 cycles of 96°C for 10 sec, 50°C for 5 sec, and 60°C for 4 min. Electrophoresis and analysis were performed under conditions similar to those in the dye primer method.

50 [0061] As a result, clone #9.31334 having the whole length of 983 bp (SEQ ID NO: 3) was obtained for 23-1#2. This sequence was found to be a novel gene comprising the region encoding 167 amino acids. This amino acid sequence exhibited about 38% homology to the bovine cystatin B, an inhibitory protein for cathepsins. Furthermore, three cDNA clones (#7.16411, #8.323, and #11.24413) were obtained for 28-1#3. Comparative studies on these nucleotide sequences revealed strong indications that #8.323 can be processed differently to produce #7.16411 and #11.24413. Clone #8.323 was 1,114 bp while clones #7.16411 and #11.24413 were 691 bp and 684 bp, respectively. Clones #7.16411 and #11.24413 represented an identical nucleotide sequence except for about 100 bp on the 5'-terminus side and were considered to encode the proteins of 131 and 126 amino acids with a difference of 20 to 30 amino acids at the N-terminus. From the deduced amino acid sequences, #7.16411 was considered to have a hydrophobic N-terminus, while #11.24413 was considered to be hydrophilic. A homology search indicated that three kinds of sequences

obtained using 28-1#3 also represented novel genes similar to #9.31334. Furthermore, a protein presumed to be encoded by #7.16411 exhibited nearly 60% homology to human SLPI (secretory leukoprotease inhibitor). Nucleotide sequences of these cDNA clones are shown in SEQ ID NO: 5 (nucleotide sequence of the clone #7.16411), SEQ ID NO: 7 (nucleotide sequence of the clone #8.323), and SEQ ID NO: 8 (nucleotide sequence of the clone #11.24413).

#### Example 6 Expression of #7.16411 and #11.24413 in various cancer cells and normal tissues

[0062] Primers which specifically amplify #7.16411 and #11.24413 were designed, and the expression of these clones in various cancer cells and normal tissues was examined by RT-PCR. PCR was performed under the conditions in which each cDNA can be quantitatively detected in IMC-HA1 cells (conditions described below in Example 7). As set of primers, "7F2" (SEQ ID NO: 35) and "28-1#3F1" (SEQ ID NO: 31) were used to amplify #7.16411, and "11F2" (SEQ ID NO: 49) and "28-1#3F1" (SEQ ID NO: 31), to amplify #11.24413. As a result, it was found that #7.16411 was able to be amplified in almost all cancer cells and normal tissues examined, though the gradation of developed color differed somewhat. In contrast, #11.24413 could be detected only in cancer cells such as the P388 and IMC-HA1 cells, and in normal tissues such as the thymus, lung, and spleen, and slightly in peripheral blood. From these results, it is highly possible that the processing to produce #7.16411 is common in the majority of normal tissues, while #11.24413 is expressed only in particular cells. #11.24413 was considered to be an important factor to impart to the IMC-HA1 cells characteristics such as the ability to rapidly invade the whole body.

#### Example 7 Expression of mouse CMAP in various cancer cell lines and normal tissues

[0063] The amino acid sequence deduced from the coding region of #9.31334 is designated mouse CMAP and is so called below.

[0064] Expression of mouse CMAP was examined in 12 different mouse cancer cell lines by reverse transcription PCR (RT-PCR)(Figure 1). Total RNA was extracted from each cell line by a method similar to that in Example 1 (1), and a complementary single-stranded cDNA was prepared similarly to that in Example 1 (2). Mouse CMAP was then detected by PCR similarly as in Example 1 (5). The reaction was comprised of 94°C for 2 min; 30 cycles of 94°C for 1 min and 68°C for 2 min; and 68°C for 5 min, using a set of primers "23-1#2F1" (SEQ ID NO: 29) and "23-1#2R1" (SEQ ID NO: 30). Preliminary examination of the dilution of a single-stranded cDNA serving as a template confirmed that sub-quantitative conditions producing a nearly linear relationship between the dilution and product yield were established for IMC-HA1 cells when CMAP was diluted 10-fold for the amplification and the control G3PDH was diluted 40-fold for the detection.

[0065] Mouse CMAP was thus clearly expressed in M-5076 cells, L5178Y cells, P388 cells, and L1210 cells in addition to IMC-HA1 cells. Anatomical observation with the naked eye and pathological analysis confirmed that all of these cell lines formed metastatic foci mainly in the liver when  $1 \times 10^5$  cells were transplanted through the tail vein of mice. B-16-BL6 cells and Colon 26 cells in which almost no expression of mouse CMAP could be confirmed strongly induced an experimental metastatic foci in the lung, but not in the liver. Furthermore, all other cell lines wherein the expression of mouse CMAP was below the detection limit were almost rejected and regressed after the transplantation through the tail vein of mice. These results indicated that the expression of mouse CMAP could be closely associated with the metastasis of cancer cells to the liver.

[0066] In addition, 15 tissues were surgically excised from two to three 5-week-old normal female CDF1 mice and disrupted using a Polytron homogenizer. The expression of mouse CMAP was confirmed by RT-PCR similarly as described above (Figure 2). Mouse CMAP was found to be expressed in the thymus, spleen, and mesenteric lymph nodes, although the expression was clearly weaker than for IMC-HA1 cells. These results indicated that mouse CMAP could also physiologically function in normal tissues, profoundly associating with the immune mechanism.

#### Example 8 Identification of human homologue of mouse CMAP

##### (1) Database Search

[0067] The present inventors searched for a human nucleotide sequence showing homology to that of mouse CMAP in the Genbank and EMBL databases with a Fast A using VMS (Version 8) software from Genetics Computer Group. The results revealed that two Expressed Sequence Tags (ESTs), N47763 and N56875, showed 75.7% and 72.0% homologies to mouse CMAP, respectively. These two ESTs hold a homologous sequence comprising 34 nucleotide residues, strongly indicating that they might be derived from human CMAP of the same origin.

## (2) Amplification of human CMAP by RT-PCR

[0068] Since mouse CMAP was expressed in the spleen among normal tissues of mice, the human spleen was chosen as the supply source of template DNA. A single-stranded cDNA was prepared from the total RNA (OriGene) (2 µg) extracted from the human spleen by a method similar to that in Example 1 (2). Based on sequences of N47763 and N56875, eight primers (SEQ ID NOs: H-CHAP-1 to 8) were designed. PCR was performed on the template single-stranded cDNA using two primers, including H-CMAP-1 and H-CMAP-2 similarly as in Example 1 (5). Reaction was carried out in the sequence 94°C for 2 min; 30 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 90 sec; and 72°C for 5 min. The thermal cycler used was a PERKIN ELMER 9600.

## (3) Sequencing

[0069] The nucleotide sequence of the cDNA product of about 0.8 kb (SEQ ID NO: 37) derived from the amplified human CMAP was directly sequenced according to a method similar to that in Example 1 (6) using primers H-CMAP-1 to 8 (SEQ ID NOs: 39 to 46). Human CMAP exhibited a 65.9% homology to mouse CMAP. In addition, human CMAP was located in the nucleotide residues from 83 to 583, and its amino acid sequence (SEQ ID NO: 38) possessed a 71.6% homology to that of mouse CMAP. Therefore, it was strongly indicated that these two proteins have approximately the same function.

## (4) Comparison of amino acid sequences of human CMAP with family 2 cystatin

[0070] Amino acid sequences of mouse and human CMAPs and human family 2 cystatin were compared by a multi-sequence comparison program, MegAlign, in the Lasergene (DNASTAR) (Figure 3). Consensus sequences of human family 2 cystatin (cystein residues at positions 121, 132, 146, and 166; glycine residue at 59; QXVXG at 103 to 107; and VFW at 153 to 155) were found to be minimally conserved in CMAPs. However, the overall homology was 30% or less in all CMAPs. In particular, sequences at the N-terminus were characteristic and long, and found to comprise many cysteine residues which may impart specificity to CMAP. Theoretically, it is highly possible that N of position 84 is glycosylated, a characteristic not observed in other sequences in its vicinity. Although CMAPs hold sequences resembling those hitherto reported for cystatins, they were found to comprise many characteristic amino acid residues, indicating the possibility of having properties differing from those of known substances.

Example 9 Decrease in cancerous metastasis potency of IMC-HA1 cells in mice due to the constitutive decrease of mouse CMAP

[0071] It is generally agreed that alterations of the nature, quality or expression level of multiple overlapped genes are required for metastasis. Therefore, it was thought to be difficult to induce a single metastasis-associated gene in cells with low metastasis potency in mouse to confirm the elevation of their metastasis ability. IMC-HA1 cells were therefore transformed with a vector in which a sequence complementary to the mouse CMAP mRNA was inserted to construct stably transformed clonal cells, which were examined to determine the change in metastasis potency in mice.

## (1) Construction of pBK-AS-CMAP vector

[0072] The pBK-CMV expression vector was treated with restriction enzymes NheI and KpnI to prepare a linear vector whose lacZ promoter and multicloning sites were removed. The insert was prepared from the total mRNA of IMC-HA1 cells by RT-PCR as the starting material. CMAP-F1 (SEQ ID NO: 47) and CHAP-R1 (SEQ ID NO: 48) were used as a set of primers. The NheI and KpnI restriction enzyme recognition sites are introduced in these primers at their termini, and their amplification product corresponds to the entire noncoding and coding regions on the 5'-side of mouse CMAP. The amplification product of about 0.5 kb was digested with restriction enzymes NheI and KpnI, purified with a QIAquick PCR Purification Kit (QIAGEN), and ligated to the above-prepared linear vector using a DNA Ligation Kit (Takara Shuzo). In this way, the pBK-AS-CMAP vector that expresses mRNA acting as the antisense against mouse CMAP in cells was constructed.

## (2) Transfer of pBK-AS-CMAP vector into IMC-HA1 cells and selection of transformants

[0073] After the pBK-AS-CMAP vector was introduced into *E. coli* competent cells and cultured on a large scale, plasmids were prepared in large amounts according to the standard method using a QIAfilter Plasmid Midi Kit (QIAGEN). The plasmid (about 50 µg) was cleaved at one site with ApaI to make it linear, purified by phenol extraction and ethanol precipitation, and dissolved in EP buffer (50 µl) (containing 25 mM Hepes (pH 7.0), 137 mM NaCl, 5 mM KCl,

0.27mM Na<sub>2</sub>HPO<sub>4</sub>, and 6mM dextrose). IMC-HA1 cells (about  $1 \times 10^8$  cells) at the logarithmic growth phase were recovered from the culture medium (100 ml) and resuspended in EP buffer to prepare a cell suspension of  $4 \times 10^7$  cells/ml. This cell suspension (450  $\mu$ l) was mixed with the plasmid solution prepared above (50  $\mu$ l), and plasmids were transfected into cells by electroporation under the conditions of 250 mV, 500  $\mu$ F, and a single pulse using a GenePulser II (Bio-Rad). After the mixture was allowed to stand at room temperature for 10 min, cells were cultured under the usual conditions. After 24-hour culturing, transformed cells were continuously treated with neomycin (0.3 mg/ml) for 2 weeks to select stable transformants. The cells were cloned by the limiting dilution method to obtain about 50 clonal cells. The cells in which the expression level of mouse CMAP was decreased were screened by RT-PCR. As a result, three lines of cell clones, 57C4, 53A9, and 53E9, were obtained. Similar procedures were also performed with pBK-CMV vector with no insert, and an 18B5 cell line was obtained as the vector control cell clone. When the expression of mouse CMAP mRNA in these cell lines was confirmed by Northern blot analyses, said mRNA was expressed in the 18B5 cell line at almost the same level as that in the IMC-HA1 cell line, but only about 49%, 32%, and 16% in 57C4, 53A9, and 53E9 cell lines. No particular changes in the proliferation and morphology during culturing were noticed in any of these cell lines.

### (3) Confirmation of metastasis potency *in vivo*

[0074] Metastasis abilities of cloned cell lines derived from IMC-HA1 cells with different expression patterns of mouse CMAP mRNA were tested in mice by three independent experiment systems. In experiment I, each cell line ( $5 \times 10^4$  cells) was subcutaneously transplanted to mice. The liver was excised on the 15th day after the transplantation and subjected to observation of appearance, weight determination, and calculation of the survival rate. Experiment II was performed in the same matter as experiment I except that on the third day after the transplantation primary tumors at the transplantation site were excised together with proximal lymph nodes. In this experiment, a model of a minute metastatic focus of IMC-HA1 cells in the liver can be prepared. Furthermore, in experiment III, cells ( $1 \times 10^3$  cells) were directly transferred through the tail vein, and the liver was excised on the 13th day after transplantation. At the same time, a diachronic study of the survival was performed. The experiment revealed that the expression of mouse CMAP mRNA was closely associated with the formation of liver metastatic focus and survival of mice (Table 1). Note 1) in the table represents "Number of mice which survived for 60 days/Number of mice tested."

Table 1

## Experiment I

Cell line	cmep mRNA(%)	Liver weight (g)				Survival days				
		mean $\pm$ SD	P value	T/C %		mean $\pm$ SD	P value	T/C %	S/T <sup>1)</sup>	
IMC-HA1	100	2.070 $\pm$ 0.409	-	173		17.4 $\pm$ 1.5	-	100	0/5	
18B5	101	2.240 $\pm$ 0.351	0.2500	187		17.6 $\pm$ 2.1	0.8825	101	0/5	
57C4	49	1.456 $\pm$ 0.109	0.0002	122		19.8 $\pm$ 1.9	0.0867	114	0/5	
53A9	32	1.262 $\pm$ 0.079	<0.0001 s	106		23.4 $\pm$ 2.3	0.0002	134	0/5	
53E9	16	1.223 $\pm$ 0.130	<0.0001 s	102		25.4 $\pm$ 3.4	<0.0001 s	146	0/5	
IMC-LE5	<5	1.140 $\pm$ 0.082	<0.0001 s	95		>59.0 $\pm$ 2.2	<0.0001 s	>339	4/5	
normal		1.196 $\pm$ 0.080	<0.0001 s	100						

## Experiment II

Cell line	cmep mRNA(%)	Liver weight (g)				Survival days				
		mean $\pm$ SD	P value	T/C %		mean $\pm$ SD	P value	T/C %	S/T <sup>1)</sup>	
IMC-HA1	100	1.506 $\pm$ 0.149	-	145		18.0 $\pm$ 0.7	-	100	0/5	
18B5	101	1.624 $\pm$ 0.479	0.3751	157		18.0 $\pm$ 1.6	1.0000	100	0/5	
57C4	49	1.047 $\pm$ 0.199	0.0020 s	101		20.6 $\pm$ 1.1	0.1600	114	0/6	
53A9	32	0.947 $\pm$ 0.144	0.0001 s	91		>30.7 $\pm$ 15.1	0.0001 s	>171	1/6	
53E9	16	1.050 $\pm$ 0.145	0.0013 s	101		>43.0 $\pm$ 18.8	<0.0001 s	>239	3/6	
IMC-LE5	<5	0.962 $\pm$ 0.063	0.0002 s	93		>53.5 $\pm$ 7.8	<0.0001 s	>297	3/6	
normal		1.036 $\pm$ 0.139	0.0010 s	100						

## Experiment III

Cell line	cmep mRNA(%)	Liver weight (g)				Survival days				
		mean $\pm$ SD	P value	T/C %		mean $\pm$ SD	P value	T/C %	S/T <sup>1)</sup>	
IMC-HA1	100	1.767 $\pm$ 0.460	-	191		14.0 $\pm$ 0.7	-	100	0/5	
18B5	101	1.940 $\pm$ 0.161	0.2036	209		13.6 $\pm$ 0.5	0.8278	97	0/5	
57C4	49	1.102 $\pm$ 0.186	<0.0001 s	119		19.2 $\pm$ 1.5	0.0086	137	0/5	
53A9	32	0.960 $\pm$ 0.094	<0.0001 s	104		21.4 $\pm$ 2.6	0.0004 s	153	0/5	
53E9	16	0.940 $\pm$ 0.061	<0.0001 s	101		>36.0 $\pm$ 20.7	<0.0001 s	257	1/5	
IMC-LE5	<5	0.949 $\pm$ 0.103	<0.0001 s	102		>60.0 $\pm$ 0.0	<0.0001 s	>429	5/5	
normal		0.927 $\pm$ 0.052	<0.0001 s	100						

[0075] It is especially noteworthy that in experiment II, one out of six mice in which 53A9 cells were transplanted and three (half) of six mice in which 53E9 cells were transplanted survived for 60 days or more without metastasis to the liver. These results suggested that the expression of mouse CMAP is profoundly associated with the formation of metastatic focus in the liver, and that the gene acts mainly after cancer cells enter the vessel.



Industrial Applicability

[0076] The present invention provides a novel protein associated with cancer metastasis and a DNA encoding said protein. This invention also provides a vector carrying said DNA, a transformant harboring said vector, and a method for preparing a recombinant protein by culturing said transformant. In addition, this invention provides DNA to be used to detect, isolate, amplify, or suppress the expression of said gene. Furthermore, this invention provides a method for screening cancer metastasis inhibitors utilizing said protein, enabling development of novel cancer metastasis inhibitors.

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## Sequence Listing

- (1) Name of Applicant: Banyu Pharmaceutical Co., Ltd.
- (2) Title of the Invention: Cancer Metastasis-associated Gene
- (3) Reference Number: B1-001PCT
- (4) Application Number:
- (5) Filing Date:
- (6) Country where the priority application was filed and the application number of the application: Japan, No. Hei 9-105333
- (7) Priority date: April 8, 1997
- (8) Number of Sequences: 49

SEQ ID NO: 1  
SEQUENCE LENGTH: 656  
SEQUENCE TYPE: nucleic acid  
STRANDEDNESS: double  
TOPOLOGY: linear  
MOLECULE TYPE: cDNA  
HYPOTHETICAL: No  
ANTISENSE: Yes  
ORIGINAL SOURCE:  
    ORGANISM: Mus Musculus  
    CELL LINE: IMC-HA1 cell

SEQUENCE DESCRIPTION: SEQ ID NO: 1

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CGGGCATCAA GAAGCTTCCC AACTACCAC CTTGTTGACT GTATCTGCTT TGCTCTGCAC	180
GTGCTCCAGT AATGTCATCA AAGGACTCTC TGCCTGCCCA CATCCTGAAT CTAGGTGTGT	240

AGCACCAGAG AATTCATTTT AGAAGAAAGT GTGAATACAG TTAATCTGGA GAGGAGGGCA 300  
 CTATATTCTC CCAGTCCAGC CCCCTGATGA CCCACTGGCA TGTTAATCTG CTCGCTGGTA 360  
 AGCAGGGCCCT GAGGATCTGA GATGGCCAGG ATGGGGTGGG AAGTAAGGAG TGGGGAAAAC 420  
 ACGAGTGGAT ATGTATCACA GCTGCAGTCT TGCTAAAGAG ACAGAAGTCA CTGGCAGAGG 480  
 AGAACAGGCA CCTCAAACT GTGGAGCCAG GGGATGACCC AGACTTCAGA GTAGCAATAT 540  
 AGAGTCCGCT TCAAGGCAGG GTTGGTTTGG AAGTCACAGT TGTCCAGTTG GTGATGCATG 600  
 GTCTTCCTGC ATGTAGTTGG GCGGATTTTC ACCTCCAGCA TTTAGTGAGG GTTAAT 656

SEQ ID NO: 2  
 SEQUENCE LENGTH: 369  
 SEQUENCE TYPE: nucleic acid  
 STRANDEDNESS: double  
 TOPOLOGY: linear  
 MOLECULE TYPE: cDNA  
 HYPOTHETICAL: No  
 ANTISENSE: Yes  
 ORIGINAL SOURCE:  
 ORGANISM: Mus Musculus  
 CELL LINE: IMC-HA1 cell

SEQUENCE DESCRIPTION: SEQ ID NO: 2  
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 AAAACAGTAA CCCTGGTCCA CAGGATCCAA GCCTGGGATA CAGGGAGCCG GGAGCCATGG 180  
 AACCCGGAGC AGGGACGCCT GGGAGCAGGG AAGTAGTCTC CAGAGCACAC CGAGCAGCAG 240  
 TCCAGAGCCG GCGCCAATGT CAGGGATCAG GCTCACATCG GGGGCAGGCA GACTTTCCCA 300  
 CATATACCCT CACAGCACTT GTATTTGCCG TCACACTGCC CGTCCCTCCA GCATTTAGTG 360  
 AGGGTTAAT 369

SEQ ID NO: 3  
 SEQUENCE LENGTH: 983  
 SEQUENCE TYPE: nucleic acid  
 STRANDEDNESS: double  
 TOPOLOGY: linear  
 MOLECULE TYPE: cDNA to mRNA  
 HYPOTHETICAL: No

ANTISENSE: No

ORIGINAL SOURCE:

ORGANISM: Mus Musculus

CELL LINE: IMC-HA1 cell

IMMEDIATE SOURCE

LIBRARY: IMC-HA1 cell-derived cDNA library

CLONE: 23-1#2

FEATURE:

NAME/KEY: CDS

LOCATION: 17..520

IDENTIFICATION METHOD: E

SEQUENCE DESCRIPTION: SEQ ID NO: 3

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	1 5 10	
25	GGC CCA CTA CAT GCT CCC TGC ATT TCC CCA GCC ATG TGG CTG GCC ATT	100
	Gly Pro Leu His Ala Pro Cys Ile Ser Pro Ala Met Trp Leu Ala Ile	
	15 20 25	
30	CTG CTT GCC CTC TGC TGC CTA ACT TCT GAC ACC CAT GGG GCA CGC CCC	148
	Leu Leu Ala Leu Cys Cys Leu Thr Ser Asp Thr His Gly Ala Arg Pro	
	30 35 40	
35	CCA GAT TTT TGT TCC AAA GAT TTG ATC TCC AGT GTG AAG CCA GGA TTC	196
	Pro Asp Phe Cys Ser Lys Asp Leu Ile Ser Ser Val Lys Pro Gly Phe	
	45 50 55 60	
	CCC AAA ACA ATA GAG ACC AAT AAC CCA GGA GTG CTT AAG GCC GCC AGG	244
	Pro Lys Thr Ile Glu Thr Asn Asn Pro Gly Val Leu Lys Ala Ala Arg	
40	65 70 75	
	CAC AGT GTG GAA AAG TTC AAC AAC TGC ACA AAT GAC ATC TTT TTG TTC	292
	His Ser Val Glu Lys Phe Asn Asn Cys Thr Asn Asp Ile Phe Leu Phe	
	80 85 90	
45	AAG GAG TCC CAT GTC AGC AAA GCC CTG GTA CAG GTG GTG AAA GGC CTG	340
	Lys Glu Ser His Val Ser Lys Ala Leu Val Gln Val Val Lys Gly Leu	
	95 100 105	
50	AAA TAT ATG CTG GAG GTG AAA ATC GGC CGA ACT ACA TGC AGG AAG ACC	388
	Lys Tyr Met Leu Glu Val Lys Ile Gly Arg Thr Thr Cys Arg Lys Thr	

110 115 120  
 5 ATG CAT CAC CAA CTG GAC AAC TGT GAC TTC CAA ACC AAC CCT GCC TTG 436  
 Met His His Gln Leu Asp Asn Cys Asp Phe Gln Thr Asn Pro Ala Leu  
 125 130 135 140  
 10 AAG CGG ACT CTA TAT TGC TAC TCT GAA GTC TGG GTC ATC CCC TGG CTC 484  
 Lys Arg Thr Leu Tyr Cys Tyr Ser Glu Val Trp Val Ile Pro Trp Leu  
 145 150 155  
 CAC AGT TTT GAG GTG CCT GTT CTC CTC TGC CAG TGA CTTCTGT CTCTTTAGCA 537  
 His Ser Phe Glu Val Pro Val Leu Leu Cys Gln  
 15 160 165  
 AGACTGCAGC TGTGATACAT ATCCACTCGT GTTTTCCCCA CTCCTTACTT TCCACCCCAT 597  
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 20 TCATCAGGGG GCTGGACTGG GAGAATATAG TGCCCTCCTC TCCAGATTAA CTGTATTAC 717  
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 GAGAGTCCTT TGATGACATT ACTGGAGCAC GTGCAGAGCA AAGCAGATAC AGTCAACAAG 837  
 GTGGTAGTGT GGAAGCTTC TTGATGCCCC ACTGCATGAG CAGATTCTGC AGCCGTCTGG 897  
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30 -SEQ ID NO: 4  
 SEQUENCE LENGTH: 167  
 SEQUENCE TYPE: amino acid  
 TOPOLOGY: linear  
 35 MOLECULE TYPE: protein  
 ORIGINAL SOURCE:  
 ORGANISM: Mus Musculus  
 CELL LINE: IMC-HA1 cell  
 40

SEQUENCE DESCRIPTION: SEQ ID NO: 4  
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 Leu Leu Ala Leu Cys Cys Leu Thr Ser Asp Thr His Gly Ala Arg Pro  
 50 30 35 40  
 Pro Asp Phe Cys Ser Lys Asp Leu Ile Ser Ser Val Lys Pro Gly Phe

55

45                      50                      55                      60  
 Pro Lys Thr Ile Glu Thr Asn Asn Pro Gly Val Leu Lys Ala Ala Arg  
 5                      65                      70                      75  
 His Ser Val Glu Lys Phe Asn Asn Cys Thr Asn Asp Ile Phe Leu Phe  
 80                      85                      90  
 10 Lys Glu Ser His Val Ser Lys Ala Leu Val Gln Val Val Lys Gly Leu  
 95                      100                      105  
 Lys Tyr Met Leu Glu Val Lys Ile Gly Arg Thr Thr Cys Arg Lys Thr  
 110                      115                      120  
 15 Met His His Gln Leu Asp Asn Cys Asp Phe Gln Thr Asn Pro Ala Leu  
 125                      130                      135                      140  
 Lys Arg Thr Leu Tyr Cys Tyr Ser Glu Val Trp Val Ile Pro Trp Leu  
 145                      150                      155  
 20 His Ser Phe Glu Val Pro Val Leu Leu Cys Gln  
 160                      165

25 SEQ ID NO: 5  
 SEQUENCE LENGTH: 691  
 SEQUENCE TYPE: nucleic acid  
 STRANDEDNESS: double  
 30 TOPOLOGY: linear  
 MOLECULE TYPE: cDNA to mRNA  
 HYPOTHETICAL: No  
 35 ANTISENSE: No  
 ORIGINAL SOURCE:  
     ORGANISM: Mus Musculus  
     CELL LINE: IMC-HA1 cell  
 40 IMMEDIATE SOURCE:  
     LIBRARY: IMC-HA1 cell-derived cDNA library  
     CLONE: 28-1#3  
 45 FEATURE:  
     NAME/KEY: CDS  
     LOCATION: 37..429  
     IDENTIFICATION METHOD: E  
 50

55 SEQUENCE DESCRIPTION: SEQ ID NO: 5

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 1 5  
 TTA CCT TTC ACG GTG CTC CTT GCT CTG GGG ATC CTG GCA CCC TGG ACT 102  
 Leu Pro Phe Thr Val Leu Leu Ala Leu Gly Ile Leu Ala Pro Trp Thr  
 10 10 15 20  
 GTG GAA GGA GGC AAA AAT GAT GCT ATC AAA ATC GGA GCC TGC CCT GCT 150  
 Val Glu Gly Gly Lys Asn Asp Ala Ile Lys Ile Gly Ala Cys Pro Ala  
 25 30 35  
 AAA AAG CCT GCC CAG TGC CTT AAG CTT GAG AAG CCA CAA TGC CGT ACT 198  
 Lys Lys Pro Ala Gln Cys Leu Lys Leu Glu Lys Pro Gln Cys Arg Thr  
 40 45 50  
 GAC TGG GAG TGC CCG GGA AAG CAG AGG TGC TGC CAA GAT GCT TGC GGT 246  
 Asp Trp Glu Cys Pro Gly Lys Gln Arg Cys Cys Gln Asp Ala Cys Gly  
 55 60 65 70  
 TCC AAG TGC GTG AAT CCT GTT CCC ATT CGC AAA CCA GTG TGG AGG AAG 294  
 Ser Lys Cys Val Asn Pro Val Pro Ile Arg Lys Pro Val Trp Arg Lys  
 75 80 85  
 CCT GGG AGG TGC GTC AAA ACT CAG GCA AGA TGT ATG ATG CTT AAC CCT 342  
 Pro Gly Arg Cys Val Lys Thr Gln Ala Arg Cys Met Met Leu Asn Pro  
 90 95 100  
 CCC AAT GTC TGC CAG AGG GAC GGG CAG TGT GAC GGC AAA TAC AAG TGC 390  
 Pro Asn Val Cys Gln Arg Asp Gly Gln Cys Asp Gly Lys Tyr Lys Cys  
 105 110 115  
 TGT GAG GGT ATA TGT GGG AAA GTC TGC CTG CCC CCG ATG TGAGCCTGAT 439  
 Cys Glu Gly Ile Cys Gly Lys Val Cys Leu Pro Pro Met  
 120 125 130  
 CCCTGACATT GGC GCCGGCT CTGGA CTG GCTCGGTGTG CTCTGGAAAC TACTTCCCTG 499  
 CTCCAGGCG TCCCTGCTCC GGGTTCCATG GCTCCCGGCT CCCTGTATCC CAGGCTTGGA 559  
 TCCTGTGGAC CAGGGTTACT GTTTTACCAC TAACATCTCC TTTTGGCTCA GCATTACCG 619  
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 AAAAAAAAAA AA 691

SEQ ID NO: 6

SEQUENCE LENGTH: 131

SEQUENCE TYPE: amino acid

TOPOLOGY: linear

MOLECULE TYPE: protein

ORIGINAL SOURCE:

ORGANISM: Mus Musculus

CELL LINE: IMC-HA1 cell

SEQUENCE DESCRIPTION: SEQ ID NO: 6

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Met Lys Ser Cys Gly Leu
1 5
Leu Pro Phe Thr Val Leu Leu Ala Leu Gly Ile Leu Ala Pro Trp Thr
10 15 20
Val Glu Gly Gly Lys Asn Asp Ala Ile Lys Ile Gly Ala Cys Pro Ala
25 30 35
Lys Lys Pro Ala Gln Cys Leu Lys Leu Glu Lys Pro Gln Cys Arg Thr
40 45 50
Asp Trp Glu Cys Pro Gly Lys Gln Arg Cys Cys Gln Asp Ala Cys Gly
55 60 65 70
Ser Lys Cys Val Asn Pro Val Pro Ile Arg Lys Pro Val Trp Arg Lys
75 80 85
Pro Gly Arg Cys Val Lys Thr Gln Ala Arg Cys Met Met Leu Asn Pro
90 95 100
Pro Asn Val Cys Gln Arg Asp Gly Gln Cys Asp Gly Lys Tyr Lys Cys
105 110 115
Cys Glu Gly Ile Cys Gly Lys Val Cys Leu Pro Pro Met
120 125 130

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SEQ ID NO: 7

SEQUENCE LENGTH: 1114

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: double

TOPOLOGY: linear

MOLECULE TYPE: cDNA

HYPOTHETICAL: No

ANTISENSE: No

ORIGINAL SOURCE:

ORGANISM: Mus Musculus

CELL LINE: IMC-HA1 cell



## IMMEDIATE SOURCE:

LIBRARY: IMC-HA1 cell-derived cDNA library

CLONE: #8.323

## SEQUENCE DESCRIPTION: SEQ ID NO: 7

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 CCCAGTGTGT CTTCTTCAAT GTAACCTCAG GACCTAGAAC AGCACCTTGC ATGTGCTCTC 240  
 15 AGGTGGTTAC TCTGATGGCC TCATGGTCCT GCCTGAAACA GAAAGTCTGC CACCTACTTC 300  
 TGTAGCAGCA AGACTCCTGT TCTGTGGCTA AGCTTCCTGC CTGTGCAAGA GCCACAGGGA 360  
 GGGGCCAAAT GCATGCCACT GGGGCCACGC CTCCTGGTAA AGACATAAAT AGTGATCCTC 420  
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 ACGGTGCTCC TTGCTCTGGG GATCCTGGCA CCCTGGACTG TGGAAGGAGG CAAAAATGAT 540  
 GCTATCAAAA TCGGAGCCTG CCCTGCTAAA AAGCCTGCCC AGTGCCTTAA GCTTGAGAAG 600  
 CCACAATGCC GTACTGACTG GGAGTGCCCG GGAAAGCAGA GGTGCTGCCA AGATGCTTGC 660  
 25 GGTTCCAAGT GCGTGAATCC TGTTCCTATT CGCAAACCAG TGTGGAGGAA GCCTGGGAGG 720  
 TCGGTCAAAA CTCAGGCAAG ATGTATGATG CTTAACCTC CCAATGTCTG CCAGAGGGAC 780  
 GGGCAGTGTG ACGGCAAATA CAAGTGCTGT GAGGGTATAT GTGGGAAAGT CTGCCTGCCC 840  
 CCGATGTGAG CCTGATCCCT GACATTGGCG CCGGCTCTGG ACTCGTGCTC GGTGTGCTCT 900  
 30 GGAAACTACT TCCCTGCTCC CAGGCGTCCC TGCTCCGGGT TCCATGGCTC CCGGCTCCCT 960  
 GTATCCCAGG CTTGGATCCT GTGGACCAGG GTTACTGTTT TACCACTAAC ATCTCCTTTT 1020  
 GGCTCAGCAT TCACCGAGCT TTAGGGAAAT GCTGTTGGAG AGCAAATAAA TAAACGCATT 1080  
 35 CATTTCTCTA TGCAAAAAA AAAAAAAAAA AAAA 1114

## SEQ ID NO: 8

SEQUENCE LENGTH: 684

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: double

TOPOLOGY: linear

MOLECULE TYPE: cDNA to mRNA

HYPOTHETICAL: No

ANTISENSE: No

ORIGINAL SOURCE:

ORGANISM: Mus Musculus

CELL LINE: IMC-HA1 cell

## ORIGINAL SOURCE:

LIBRARY: IMC-HA1 cell-derived cDNA library

CLONE: 28-1#3

## FEATURE:

NAME/KEY: CDS

LOCATION: 40..420

IDENTIFICATION METHOD: E

## SEQUENCE DESCRIPTION: SEQ ID NO: 8

TGTATTAAAT GATCTGGAAG CACCAGAGGC CAGTTTGGG ATG CCA AAC CCC TAC CTA 57

Met Pro Asn Pro Tyr Leu

1

5

ACC AGA AGA AGA GAA GAA AGG CCA CTG CCG AGG TCA CTT CCA GTA CTT 105

Thr Arg Arg Arg Glu Glu Arg Pro Leu Pro Arg Ser Leu Pro Val Leu

10

15

20

GGA GAT GCT ATC AAA ATC GGA GCC TGC CCT GCT AAA AAG CCT GCC CAG 153

Gly Asp Ala Ile Lys Ile Gly Ala Cys Pro Ala Lys Lys Pro Ala Gln

25

30

35

TGC CTT AAG CTT GAG AAG CCA CAA TGC CGT ACT GAC TGG GAG TGC CCG 201

Cys Leu Lys Leu Glu Lys Pro Gln Cys Arg Thr Asp Trp Glu Cys Pro

40

45

50

GGA AAG CAG AGG TGC TGC CAA GAT GCT TGC GGT TCC AAG TGC GTG AAT 249

Gly Lys Gln Arg Cys Cys Gln Asp Ala Cys Gly Ser Lys Cys Val Asn

55

60

65

70

CCT GTT CCC ATT CGC AAA CCA GTG TGG AGG AAG CCT GGG AGG TGC GTC 297

Pro Val Pro Ile Arg Lys Pro Val Trp Arg Lys Pro Gly Arg Cys Val

75

80

85

AAA ACT CAG GCA AGA TGT ATG ATG CTT AAC CCT CCC AAT GTC TGC CAG 345

Lys Thr Gln Ala Arg Cys Met Met Leu Asn Pro Pro Asn Val Cys Gln

90

95

100

AGG GAC GGG CAG TGT GAC GGC AAA TAC AAG TGC TGT GAG GGT ATA TGT 393

Arg Asp Gly Gln Cys Asp Gly Lys Tyr Lys Cys Cys Glu Gly Ile Cys

105

110

115

GGG AAA GTC TGC CTG CCC CCG ATG TGAGCCTGAT CCCTGACATT GGCGCCGGCT 447

Gly Lys Val Cys Leu Pro Pro Met

120

125

CTGGACTCGT GCTCGGTGTG CTCTGAAAC TACTTCCCTG CTCCCAGGCG TCCCTGCTCC 507  
GGGTTCCATG GCTCCCGGCT CCTGTATCC CAGGCTTGA TCCTGTGGAC CAGGGTTACT 567  
GTTTTACCAC TAACATCTCC TTTTGGCTCA GCATTACCG AGCTTTAGGG AAATGCTGTT 627  
GGAGAGCAAA TAAATAAACG CATTCAATTC TCTATGCAAA AAAAAAAAAA AAAAAAA 684

SEQ ID NO: 9

SEQUENCE LENGTH: 126

SEQUENCE TYPE: amino acid

**TOPOLOGY:** linear

**MOLECULE TYPE:** protein

ORIGINAL SOURCE:

**ORGANISM:** Mus Musculus

CELL LINE: IMC-HA1 cell

SEQUENCE DESCRIPTION: SEQ ID NO: 9

Met Pro Asn Pro Tyr Leu

1

5

Thr Arg Arg Arg Glu Glu Arg Pro Leu Pro Arg Ser Leu Pro Val Leu

10

15

20

Gly Asp Ala Ile Lys Ile Gly Ala Cys Pro Ala Lys Lys Pro Ala Gln

25

30

35

Cys Leu Lys Leu Glu Lys Pro Gln Cys Arg Thr Asp Trp Glu Cys Pro

40

45

50

Gly Lys Gln Arg Cys Cys Gln Asp Ala Cys Gly Ser Lys Cys Val Asn

55

60

65

70

Pro Val Pro Ile Arg Lys Pro Val Trp Arg Lys Pro Gly Arg Cys Val

75

80

85

Lys Thr Gln Ala Arg Cys Met Met Leu Asn Pro Pro Asn Val Cys Gln

90

95

100

Arg Asp Gly Gln Cys Asp Gly Lys Tyr Lys Cys Cys Glu Gly Ile Cys

105

110

115

Gly Lys Val Cys Leu Pro Pro Met

120

125

SEQ ID NO: 10

SEQUENCE LENGTH: 25

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO: 10

ATTAACCCTC ACTAAATGCT GGGGA

25

SEQ ID NO: 11

SEQUENCE LENGTH: 25

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO: 11

ATTAACCCTC ACTAAATGCT GGAGG

25

SEQ ID NO: 12

SEQUENCE LENGTH: 25

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO: 12

ATTAACCCTC ACTAAATGCT GGTGG

25

SEQ ID NO: 13

SEQUENCE LENGTH: 25

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO: 13

ATTAACCCTC ACTAAATGCT GGTAG

25

SEQ ID NO: 14

SEQUENCE LENGTH: 26

SEQUENCE TYPE: nucleic acid  
STRANDEDNESS: single  
5 TOPOLOGY: linear  
MOLECULE TYPE: synthetic DNA  
SEQUENCE DESCRIPTION: SEQ ID NO: 14  
10 ATTAACCCTC ACTAAAGATC TGA CTG

26

SEQ ID NO: 15  
SEQUENCE LENGTH: 25  
15 SEQUENCE TYPE: nucleic acid  
STRANDEDNESS: single  
TOPOLOGY: linear  
20 MOLECULE TYPE: synthetic DNA  
SEQUENCE DESCRIPTION: SEQ ID NO: 15  
ATTAACCCTC ACTAAATGCT GGGTG

25

25 SEQ ID NO: 16  
SEQUENCE LENGTH: 25  
SEQUENCE TYPE: nucleic acid  
STRANDEDNESS: single  
30 TOPOLOGY: linear  
MOLECULE TYPE: synthetic DNA  
SEQUENCE DESCRIPTION: SEQ ID NO: 16  
35 ATTAACCCTC ACTAAATGCT GTATG

25

SEQ ID NO: 17  
SEQUENCE LENGTH: 25  
40 SEQUENCE TYPE: nucleic acid  
STRANDEDNESS: single  
TOPOLOGY: linear  
45 MOLECULE TYPE: synthetic DNA  
SEQUENCE DESCRIPTION: SEQ ID NO: 17  
ATTAACCCTC ACTAAATGGA GCTGG

25

50 SEQ ID NO: 18  
SEQUENCE LENGTH: 25  
55

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO: 18

ATTAACCCTC ACTAAATGTG GCAGG

25

SEQ ID NO: 19

SEQUENCE LENGTH: 25

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO: 19

ATTAACCCTC ACTAAAGCAC CGTCC

25

SEQ ID NO: 20

SEQUENCE LENGTH: 30

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO: 20

CATTATGCTG AGTGATATCT TTTTITTTAA

30

SEQ ID NO: 21

SEQUENCE LENGTH: 30

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO: 21

CATTATGCTG AGTGATATCT TTTTITTTAC

30

SEQ ID NO: 22

SEQUENCE LENGTH: 30

STRANDEDNESS: single  
5 TOPOLOGY: linear  
MOLECULE TYPE: synthetic DNA  
SEQUENCE DESCRIPTION: SEQ ID NO: 22  
CATTATGCTG AGTGATATCT TTTTTTTAG 30

10  
SEQ ID NO: 23  
SEQUENCE LENGTH: 30  
15 SEQUENCE TYPE: nucleic acid  
STRANDEDNESS: single  
TOPOLOGY: linear  
MOLECULE TYPE: synthetic DNA  
20 SEQUENCE DESCRIPTION: SEQ ID NO: 23  
CATTATGCTG AGTGATATCT TTTTTTTTCA 30

25  
SEQ ID NO: 24  
SEQUENCE LENGTH: 30  
SEQUENCE TYPE: nucleic acid  
STRANDEDNESS: single  
30 TOPOLOGY: linear  
MOLECULE TYPE: synthetic DNA  
SEQUENCE DESCRIPTION: SEQ ID NO: 24  
CATTATGCTG AGTGATATCT TTTTTTTTCC 30

35  
SEQ ID NO: 25  
SEQUENCE LENGTH: 30  
40 SEQUENCE TYPE: nucleic acid  
STRANDEDNESS: single  
TOPOLOGY: linear  
MOLECULE TYPE: synthetic DNA  
45 SEQUENCE DESCRIPTION: SEQ ID NO: 25  
CATTATGCTG AGTGATATCT TTTTTTTTCG 30

50  
SEQ ID NO: 26  
SEQUENCE LENGTH: 30  
STRANDEDNESS: single

55

5 TOPOLOGY: linear  
MOLECULE TYPE: synthetic DNA  
SEQUENCE DESCRIPTION: SEQ ID NO: 26  
CATTATGCTG AGTGATATCT TTTTTTTTGA 30

10 SEQ ID NO: 27  
SEQUENCE LENGTH: 30  
SEQUENCE TYPE: nucleic acid  
STRANDEDNESS: single  
15 TOPOLOGY: linear  
MOLECULE TYPE: synthetic DNA  
SEQUENCE DESCRIPTION: SEQ ID NO: 27  
20 CATTATGCTG AGTGATATCT TTTTTTTTGC 30

25 SEQ ID NO: 28  
SEQUENCE LENGTH: 30  
SEQUENCE TYPE: nucleic acid  
STRANDEDNESS: single  
30 TOPOLOGY: linear  
MOLECULE TYPE: synthetic DNA  
SEQUENCE DESCRIPTION: SEQ ID NO: 28  
CATTATGCTG AGTGATATCT TTTTTTTTGG 30

35 SEQ ID NO: 29  
SEQUENCE LENGTH: 20  
SEQUENCE TYPE: nucleic acid  
STRANDEDNESS: single  
40 TOPOLOGY: linear  
MOLECULE TYPE: synthetic DNA  
SEQUENCE DESCRIPTION: SEQ ID NO: 29  
45 CAGAATCTGC TCATGCAGTC 20

50 SEQ ID NO: 30  
SEQUENCE LENGTH: 20  
SEQUENCE TYPE: nucleic acid  
STRANDEDNESS: single  
55



5 TOPOLOGY: linear  
MOLECULE TYPE: synthetic DNA  
SEQUENCE DESCRIPTION: SEQ ID NO: 30  
CACTCCTTAC TTCCACCCC 20

10 SEQ ID NO: 31  
SEQUENCE LENGTH: 23  
SEQUENCE TYPE: nucleic acid  
STRANDEDNESS: single  
15 TOPOLOGY: linear  
MOLECULE TYPE: synthetic DNA  
SEQUENCE DESCRIPTION: SEQ ID NO: 31  
20 AACAGCATTT CCCTAAAGCT CGG 23

25 SEQ ID NO: 32  
SEQUENCE LENGTH: 23  
SEQUENCE TYPE: nucleic acid  
STRANDEDNESS: single  
30 TOPOLOGY: linear  
MOLECULE TYPE: synthetic DNA  
SEQUENCE DESCRIPTION: SEQ ID NO: 32  
TGGAAACTAC TTCCTGCTC CCA 23

35 SEQ ID NO: 33  
SEQUENCE LENGTH: 20  
SEQUENCE TYPE: nucleic acid  
STRANDEDNESS: single  
40 TOPOLOGY: linear  
MOLECULE TYPE: synthetic DNA  
SEQUENCE DESCRIPTION: SEQ ID NO: 33  
45 AATTAAACCCT CACTAAAGGG 20

50 SEQ ID NO: 34  
SEQUENCE LENGTH: 22  
SEQUENCE TYPE: nucleic acid  
STRANDEDNESS: single  
55

5 TOPOLOGY: linear  
 MOLECULE TYPE: synthetic DNA  
 SEQUENCE DESCRIPTION: SEQ ID NO: 34  
 GTAATACGAC TCACTATAGG GC 22

10 SEQ ID NO: 35  
 SEQUENCE LENGTH: 26  
 SEQUENCE TYPE: nucleic acid  
 15 STRANDEDNESS: single  
 TOPOLOGY: linear  
 MOLECULE TYPE: synthetic DNA  
 7F2  
 20 SEQUENCE DESCRIPTION: SEQ ID NO: 35  
 CTCGGGACTG GTCATCAGAG CTCCCC 26

25 SEQ ID NO: 36  
 SEQUENCE LENGTH: 24  
 SEQUENCE TYPE: nucleic acid  
 STRANDEDNESS: single  
 30 TOPOLOGY: linear  
 MOLECULE TYPE: synthetic DNA  
 SEQUENCE DESCRIPTION: SEQ ID NO: 37  
 GACTGTATCT GCTTTGCTCT GCAC 24

35 SEQ ID NO: 37  
 SEQUENCE LENGTH: 800  
 40 SEQUENCE TYPE: nucleic acid  
 STRANDEDNESS: double  
 TOPOLOGY: linear  
 MOLECULE TYPE: cDNA to mRNA  
 45 HYPOTHETICAL:  
 ANTISENSE: No  
 ORIGINAL SOURCE:  
 50 ORGANISM: Mus Musculus  
 CELL LINE: IMC-HA1 cell  
 FEATURE:

55

NAME/KEY: CDS

LOCATION: 83..583

IDENTIFICATION METHOD : E

SEQUENCE DESCRIPTION: SEQ ID NO: 37

ACAGACCACT GCCCCACCT GCCCTGCGCC ATCTACCCAA GAAGGCTCGG CACGGGCACC 60

AACCACTGCC TCCAAGTGCC CC ATG CTG CCT GAG AAG GCA CTG CAC GGC CAC 112

Met Leu Pro Glu Lys Ala Leu His Gly His

**1                      5                      10**

CCC CAA CTG CCC CGC ACT GTC CCT ACC CGG GCA GCC ATG CGA GCG GCT 160

Pro Gln Leu Pro Arg Thr Val Pro Thr Arg Ala Ala Met Arg Ala Ala

**15**                      **20**                      **25**

GGA ACT CTG CTG GCC TTC TGC TGC CTG GTC TTG AGC ACC ACT GGG GGC 208

Gly Thr Leu Leu Ala Phe Cys Cys Leu Val Leu Ser Thr Thr Gly Gly

**30                      35                      40**

CCT TCC CCA GAT ACT TGT TCC CAG GAC CTT AAC TCA CGT GTG AAG CCA 256

Pro Ser Pro Asp Thr Cys Ser Gln Asp Leu Asn Ser Arg Val Lys Pro

**45                      50                      55**

GGA TTT CCT AAA ACA ATA AAG ACC AAT GAC CCA GGA GTC CTC CAA GCA 304

Gly Phe Pro Lys Thr Ile Lys Thr Asn Asp Pro Gly Val Leu Gln Ala

**60                      65                      70**

CCC-AGA TAC AGT GTT GAA AAG TTC AAC AAC TGC ACG AAC GAC ATG TTC 352

Ala Arg Tyr Ser Val Glu Lys Phe Asn Asn Cys Thr Asn Asp Met Phe

75                      80                      85                      90

TTG TTC AAG GAG TCC CGC ATC ACA AGG GCC CTA GTT CAG ATA GTG AAA 400

Leu Phe Lys Glu Ser Arg Ile Thr Arg Ala Leu Val Gln Ile Val Lys

**95                      100                      105**

GGC CTG AAA TAT ATG CTC GAG GTG GAA ATT GGC AGA ACT ACC TGC AAG 448

Gly Leu Lys Thr Met Leu Glu Val Glu Ile Gly Arg Thr Thr Cys Lys

**110**

AAA AAC CAG CAC CTG CGT CTG GAT GAC TGT GAC TTC CAA ACC AAC CAC 496

Lys Asn Gln His Leu Arg Leu Asp Asp Cys Asp Phe Gln Thr Asn His

**125**

ACC TTG AAG CAG ACT CTG AGC TGC TAC TCT GAA GTC TGG GTC GTG CCC 544

Thr Leu Lys Gln Thr Leu Ser Cys Tyr Ser Glu Val Trp Val Val Pro

140 145 150

TGG CTC CAG CAC TTC GAG GTG CCT GTT CTC CGT TGT CAC TGACCCCCGC 593  
 Trp Leu Gln His Phe Glu Val Pro Val Leu Arg Cys His  
 155 160 165  
 CTCTTCAGCA AGACCACAGC CATGACAAAC ACCAGGATGC ATGCTCCTTG TCCCCTCCCA 653  
 CCGGCCTCAT GACCCAGCCT CACAGACCCT CTCAGGCCTC TGACGAGTGA GCGGGTGAAG 713  
 10 TGCCACTGGG TCACCGCAGG GCAGCTGGA TGGCAGCATG GTAGCGCCTC CTAACAGATT 773  
 AAATAGATCA CATTGCTTC TAAAATT 800

SEQ ID NO: 38

SEQUENCE LENGTH: 167

SEQUENCE TYPE: amino acid

TOPOLOGY: linear

MOLECULE TYPE: protein

ORIGINAL SOURCE:

ORGANISM: Mus Musculus

CELL LINE: IMC-HA1 cell

SEQUENCE DESCRIPTION: SEQ ID NO: 38

Met Leu Pro Glu Lys Ala Leu His Gly His  
 1 5 10  
 Pro Gln Leu Pro Arg Thr Val Pro Thr Arg Ala Ala Met Arg Ala Ala  
 15 20 25  
 Gly Thr Leu Leu Ala Phe Cys Cys Leu Val Leu Ser Thr Thr Gly Gly  
 30 35 40  
 Pro Ser Pro Asp Thr Cys Ser Gln Asp Leu Asn Ser Arg Val Lys Pro  
 45 50 55  
 Gly Phe Pro Lys Thr Ile Lys Thr Asn Asp Pro Gly Val Leu Gln Ala  
 60 65 70  
 Ala Arg Tyr Ser Val Glu Lys Phe Asn Asn Cys Thr Asn Asp Met Phe  
 75 80 85 90  
 Leu Phe Lys Glu Ser Arg Ile Thr Arg Ala Leu Val Gln Ile Val Lys  
 95 100 105  
 Gly Leu Lys Thr Met Leu Glu Val Glu Ile Gly Arg Thr Thr Cys Lys  
 110 115 120  
 Lys Asn Gln His Leu Arg Leu Asp Asp Cys Asp Phe Gln Thr Asn His  
 125 130 135

Thr Leu Lys Gln Thr Leu Ser Cys Tyr Ser Glu Val Trp Val Val Pro  
 140 145 150  
 5 Trp Leu Gln His Phe Glu Val Pro Val Leu Arg Cys His  
 155 160 165

10 SEQ ID NO: 39  
 SEQUENCE LENGTH: 21  
 SEQUENCE TYPE: nucleic acid  
 STRANDEDNESS: single  
 15 TOPOLOGY: linear  
 MOLECULE TYPE: synthetic DNA  
 SEQUENCE DESCRIPTION: SEQ ID NO: 39  
 20 ACAGACACTG CCCCCACCTG C

21

SEQ ID NO: 40  
 SEQUENCE LENGTH: 22  
 25 SEQUENCE TYPE: nucleic acid  
 STRANDEDNESS: single  
 TOPOLOGY: linear  
 MOLECULE TYPE: synthetic DNA  
 30 SEQUENCE DESCRIPTION: SEQ ID NO: 40  
 AATTTTAGAA GCAAATGTGA TC

22

35 SEQ ID NO: 41  
 SEQUENCE LENGTH: 23  
 SEQUENCE TYPE: nucleic acid  
 STRANDEDNESS: single  
 40 TOPOLOGY: linear  
 MOLECULE TYPE: synthetic DNA  
 SEQUENCE DESCRIPTION: SEQ ID NO: 41  
 45 ACATGTCGTT CGTGCAGTTG TTG

23

50 SEQ ID NO: 42  
 SEQUENCE LENGTH: 22  
 SEQUENCE TYPE: nucleic acid  
 STRANDEDNESS: single

55

TOPOLOGY: linear

MOLECULE TYPE: synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO: 42

AATTGGCAGA ACTACCTGCA AG

22

SEQ ID NO: 43

SEQUENCE LENGTH: 22

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO: 43

GGTCTTGCTG AAGAGGCGGG GG

22

SEQ ID NO: 44

SEQUENCE LENGTH: 22

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO: 44

CACTGTCCCT ACCCGGGCAG CC

22

SEQ ID NO: 45

SEQUENCE LENGTH: 23

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: synthetic DNA

SEQUENCE DESCRIPTION: SEQ ID NO: 45

ATCTACCCAA GAAGGCTCGG CAC

23

SEQ ID NO: 46

SEQUENCE LENGTH: 23

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

5 TOPOLOGY: linear  
 MOLECULE TYPE: synthetic DNA  
 SEQUENCE DESCRIPTION: SEQ ID NO: 46  
 TCTGTTAGGA GCGCTACCA TGC 23

10 SEQ ID NO: 47  
 SEQUENCE LENGTH: 35  
 SEQUENCE TYPE: nucleic acid  
 15 STRANDEDNESS: single  
 TOPOLOGY: linear  
 MOLECULE TYPE: synthetic DNA  
 20 SEQUENCE DESCRIPTION: SEQ ID NO: 47  
 AATTCGGTAC CAGCTGAAGC TACCCACCA TGCCC 35

25 SEQ ID NO: 48  
 SEQUENCE LENGTH: 34  
 SEQUENCE TYPE: nucleic acid  
 STRANDEDNESS: single  
 30 TOPOLOGY: linear  
 MOLECULE TYPE: synthetic DNA  
 SEQUENCE DESCRIPTION: SEQ ID NO: 48  
 35 AGTCGCTAGC AGAGGAGAAC AGGCACCTCA AAAC 34

40 SEQ ID NO: 49  
 SEQUENCE LENGTH: 24  
 SEQUENCE TYPE: nucleic acid  
 STRANDEDNESS: single  
 TOPOLOGY: linear  
 45 MOLECULE TYPE: synthetic DNA  
 SEQUENCE DESCRIPTION: SEQ ID NO: 49  
 TGTATTAAT GATCTGGAAG CACC 24

50

55 Claims

1. A protein comprising any one of the amino acid sequences set forth in SEQ ID NOs: 4, 6, 9, or 38, or a protein comprising any one of said amino acid sequences having substitution, deletion, or addition of one or more amino acids

and having cancer metastasis potency.

2. A protein encoded by DNA which hybridizes with DNA comprising any one of the nucleotide sequences set forth in SEQ ID NOs: 3, 5, 7, 8, or 37, said protein having cancer metastasis potency.

3. A DNA encoding the protein according to claim 1.

4. The DNA according to claim 3, comprising any one of the nucleotide sequences set forth in SEQ ID NOs: 3, 5, 7, 8, or 37.

5. A DNA hybridizing with a DNA comprising any one of the nucleotide sequences set forth in SEQ ID NOs: 3, 5, 7, 8, or 37, encoding a protein having cancer metastasis potency.

6. A vector carrying the DNA according to any one of claims 3 to 5.

7. A transformant harboring the vector according to claim 6.

8. A method for preparing the protein according to claim 1 or 2, comprising culturing the transformant set forth in claim 7.

9. A DNA specifically hybridizing with the DNA according to any one of claims 3 to 5, comprising at least 15 nucleotide residues.

10. An antisense DNA against the DNA according to any one of claims 3 to 5 or a portion thereof.

11. An antibody binding to the protein according to claim 1 or 2.

12. A method for screening a compound having cancer metastasis inhibitory ability, comprising steps of:

- (a) contacting a test sample with the protein according to claim 1 or 2, and
- (b) selecting compounds having the activity to bind to the protein according to claim 1 or 2.

13. A method for screening compounds having cancer metastasis inhibitory ability, comprising steps of:

- (a) contacting test samples with cells expressing the protein according to claim 1 or 2,
- (b) detecting the expression level of the protein according to claim 1 or 2 in cells contacted with test samples using the antibody set forth in claim 11, and
- (c) selecting a compound which reduces the expression level of the protein according to claim 1 or 2 as compared with that in cells not contacted with test samples.



Figure 1

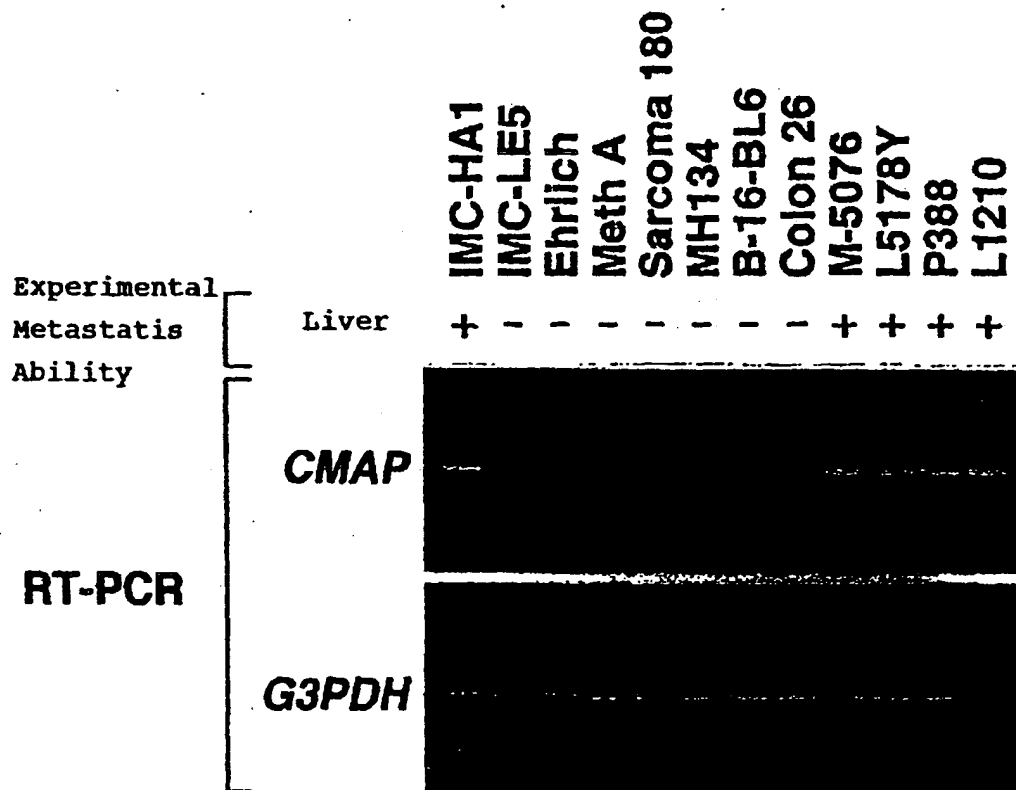


Figure 2

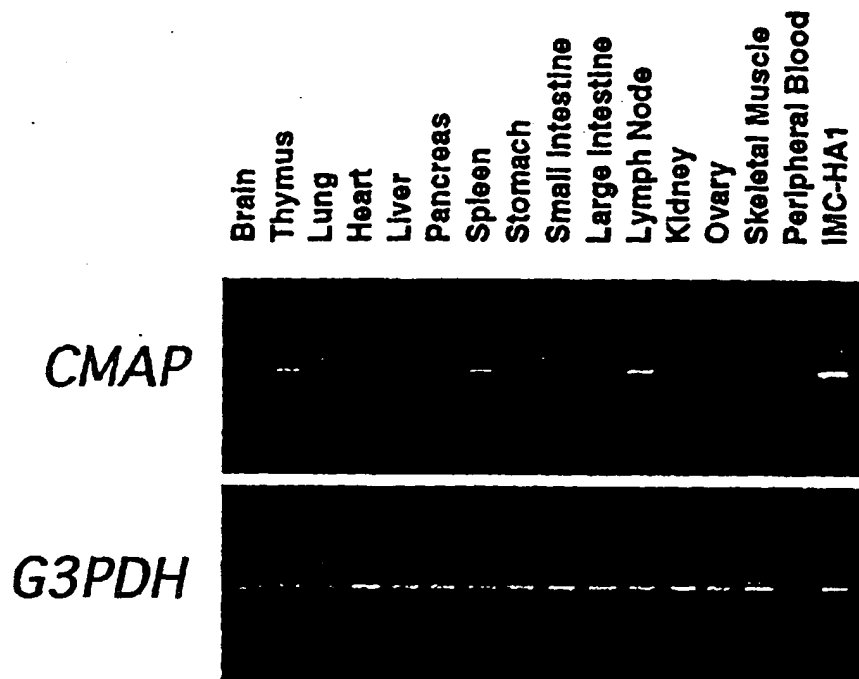


Figure 3

mouse C9AP	M P S W S C T C R A L G P L H A P C I S P A N W L A I L L A L C C L T S D T H G A R P P D F C S K D L I S S V K P C P 60	117
human C9AP	M L P E K A L H G H P Q L P R T V P T R A A K R A A G T L L A F C C L V L S T T G C P S P D T C S Q D L N S R V K P C P 60	117
human cystatin M	M A R - - - - - S N P G A L G A L V A F - - - - - C L L A L P R D A - K A R P - - - - - Q E R M Y - G E 37	118
human cystatin C	M A G - - - - - P L R A P E L L L A I L A V - - - - - A L A V S P A A G S S P G K - - - - - P P R L V Q G P 39	118
human cystatin D	M H M - - - - - P L R A P E L L L A I L A V - - - - - A L A V S P A A G S S P G K - - - - - P P R L V Q G P 39	118
human cystatin SN	M A Q - - - - - H L S T L L L L L A T L A V - - - - - A L A W S P K E E D - - - - - Q S A S A - - - - - Q S R T L A G G 33	118
human cystatin SA	M A M - - - - - P L C T L L L L L A T L A V - - - - - A L A W S P K E E D - - - - - Q S A S A - - - - - Q S R T L A G G 33	118
human cystatin S	M A R - - - - - P L C T L L L L L A T L A G - - - - - A L A S S K K E N - - - - - R I I P G G 33	118
mouse C9AP	P K T I E T N P G V L K A A R H S V E K P N N C - T N D I P L F K E S H S X A L V Q V N K Q L K Y M L E V K I G R T 119	119
human C9AP	P K T I K T N D P Q V L Q A A R Y S V E K P N N C - T N D M P L F K E S R I T R A L V Q I V K Q L K Y M L E V K I G R T 119	119
human cystatin M	L R D L S P D P Q V L Q A A R Y S V E K P N N C - T N D M P L F K E S R I T R A L V Q I V K Q L K Y M L E V K I G R T 119	119
human cystatin C	M - D A S Y E E G V R R A L D P A V Q E Y N K A - S N D M Y H S R A L Q V V R A K Q I V A Q V N Y F L D V E L G R T 97	119
human cystatin D	I H A T D L N D K S V Q R A L D P A I S E Y N K A - T N D D E Y Y R R P L R V L R A R Q Q V Q Q I V C G V N Y F N V K P G R T 93	119
human cystatin SN	I Y N A D L N D E R V Q R A L H F V I S E Y N K A - T E D E Y Y R R P L R V L R A R Q Q V Q Q I V C G V N Y F F D V E V G R T 92	119
human cystatin SA	I Y D A D L N D E R V Q R A L H F V I S E Y N K A - T E D E Y Y R R P L R V L R A R Q Q V Q Q I V C G V N Y F F D V E V G R T 92	119
human cystatin S	I Y D A D L N D E R V Q R A L H F V I S E Y N K A - T E D E Y Y R R P L R V L R A R Q Q V Q Q I V C G V N Y F F D V E V G R T 92	119
mouse C9AP	T C R A T H - - - - - H H Q L D N C D F Q T N D A L K R T L Y C Y S E V M V V I P W L H S P E V P V L L C Q 167	167
human C9AP	T C K K N Q - - - - - H L R L D D C D E Q T N H T L K Q T L S C Y S E V M V V I P W L H S P E V P V L L C Q 167	167
human cystatin M	D C R K T R V T G D H V D L T C C P L A A Q Q - Q E K L R C D F E V L V V P W L Q H P E V P V L L C Q 167	167
human cystatin C	T C T K S - - - - - Q P N L D N C P F N D Q P K L K E E E C S P O I N E V P W E D K I S L L N Y K C R K V 146	146
human cystatin D	I C T K S - - - - - Q P N L D T C A F H E Q P E L Q K K Q L C S P F I Y E V P W E D R N S L V N S R C Q E S 142	142
human cystatin SN	I C T K S - - - - - Q P N L D T C A F H E Q P E L Q K K Q L C S P F I Y E V P W E D R N S L V N S R C Q E S 141	141
human cystatin SA	I C T K S - - - - - Q P N L D T C A F H E Q P E L Q K K Q L C S P F I Y E V P W E D R N S L V N S R C Q E S 141	141
human cystatin S	I C T K S - - - - - Q P N L D T C A F H E Q P E L Q K K Q L C S P F I Y E V P W E D R N S L V N S R C Q E S 141	141

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP98/01592

## A. CLASSIFICATION OF SUBJECT MATTER

Int.Cl<sup>6</sup> C12N15/12, C12P21/02, C12N5/10, C07K14/47, C07K16/18, G01N33/53, G01N33/574

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int.Cl<sup>6</sup> C12N15/12, C12P21/02, C12N5/10

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
BIOSIS, DDBJ/Genbank/EMBL

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X/Y	Cell, 88, 1997, Fen-yu Jin et al., "Secretory leukocyte protease inhibitor: a macrophage product induced by and antagonistic to bacterial lipopolysaccharide" p.417-426	5-9, 11/10
PX/PA	Proceedings of the American Association for Cancer Research Annual Meeting, 38(0), 1997, Morita M et al., "Isolation of two metastasis-related candidate genes from a liver-metastatic murine carcinoma, IMC-HM" 546.	3-5/1, 2, 6-13
A	Japanese Journal of Cancer Research, 87(5), 1996, Arakawa H et al., "Antimetastatic effect of a novel indolocarbazole (NB-506) on IMC-HM murine tumor cells metastasized to the liver" p.518-523	1-13
Y	Science, 261, 1993, C.A. Stein et al., "Antisense oligonucleotides as therapeutic agents-Is the bullet really magical?" p.1004-1012	10

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

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Date of the actual completion of the international search  
July 3, 1998 (03. 07. 98)Date of mailing of the international search report  
July 14, 1998 (14. 07. 98)Name and mailing address of the ISA/  
Japanese Patent Office

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Telephone No.

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